REMARKS

Applicants respectfully request reconsideration of the present application in view of the following remarks.

I. Status of the Claims

Claims 27 and 29 are currently pending in the application. Claims 1-26, 28 and 30-45 were previously cancelled without prejudice to or disclaimer of the subject matter therein. No claims are amended or added.

II. The Rejections Under 35 U.S.C. § 112, First Paragraph

A. Written Description

The Office Action, at pages 2-3, rejects claim 29 under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Office Action states that Applicants do not disclose a representative number of promoter sequences from gymnosperm 4CL genes and fail to disclose a structure/function relationship for the 4CL promoters. According to the Office Action, Applicants do not disclose if all gymnosperm 4CL promoters have the same expression profile. Applicants respectfully traverse the rejection.

The description in the present application provides extensive disclosure of the 4CL1B promoter. *See* pages 9-10 and Example 5 in the specification.

Moreover, the promoter function of the claimed 4CL1B sequence, SEQ ID NO: 10, is further evidenced by comparison with the sequence of the *Pinus radiata* 4CL promoter, known to cause GUS expression in a pattern similar to the GUS expression exhibited by the 4CL1B promoter. The alignment of the 4CL1B promoter and the *Pinus radiata* 4CL promoter is

attached herein as Exhibit A. The two sequences show great similarity in several regions and are over 95% identical in the 632-1254, 1404-1524 and 1541-2221 regions of the 4CL1B promoter, corresponding to the 153-771, 825-945 and 962-1642 regions of the *Pinus radiata* 4CL promoter, respectively. The *Pinus radiata* 4CL promoter sequence is approximately 500 basepairs shorter than the 4CL1B promoter sequence, and it is likely a variant showing several deletions.

The comparison of the two sequences also enables the identification of the location of the start site of transcription, the TATA box and the CAAT box at positions 2110, 2093 and 2067 of the 4CL1B promoter, respectively, corresponding to positions 1531, 1513 and 1488 in the *Pinus radiata* 4CL promoter. Thus, a person skilled in the art would understand from the disclosure the functional regions in the promoter that are essential for the promoter's expression profile. Accordingly, the promoter sequence of the invention is adequately described in the specification and indicates that the inventors had complete possession of the claimed invention.

For at least the reasons stated above, the rejection is improper. Applicants request reconsideration and withdrawal of the rejection.

B. Enablement

The Office Action, at pages 3-4, rejects claims 27 and 29 under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Specifically, the Office Action states that Applicants have not disclosed if SEQ ID NO: 10 comprises the necessary elements essential for promoter activity, and whether the isolated promoter has any promoter activity at all. Applicants respectfully traverse the rejection.

As stated above, the promoter activity of the 4CL1B promoter is demonstrated by the close correlation with the *Pinus radiata* 4CL promoter, the GUS expression of which is similar

in pattern to the GUS expression of the 4CL1B promoter. Furthermore, Applicants submit herewith a publication by Goicoechea *et al.* (*The Plant Journal 43*: 553-567 (2005)), attached as Exhibit B, that shows that the MYB2 transcription factor isolated from *Eucalyptus gunnii* specifically binds to cis-regulatory regions in two lignin biosynthetic genes through a G(G/T)T(A/T)GGT(A/G) binding site. The binding site GGTAGGTA appears twice in SEQ ID NO: 10, thus confirming that the sequence claimed in the present application comprises the necessary elements essential for promoter binding and activity.

Clearly, the specification provides extensive information to enable the person skilled in the art to make and/or use the invention claimed in the present application. The person of skill in the art, reading the specification, can readily identify the sequences described in the claims and make the sequences using techniques provided in the specification or known to one of skill in the art. The sequences can then be tested using the procedures outlined in the specification and enabling for the many different experimental procedures described in the application. Thus, the claimed invention is fully enabled. Withdrawal of the rejection is therefore respectfully requested.

III. The Rejection Under 35 U.S.C. § 102(b)

The Office Action, at page 4, rejects claim 29 under 35 U.S.C. § 102(b) as allegedly being anticipated by Voo *et al.* (*Plant Physiol. 108*: 85-97 (1995)) ("Voo"). The Office Action states that Voo discloses a promoter sequence 5' to the ATG from a 4CL gene isolated from loblolly pine, comprising 142 base pairs that exhibit 100% sequence identity to bases 515 to 656 of SEQ ID NO: 10. Applicants respectfully traverse the rejection.

Claim 29 is directed to an isolate DNA that comprises the promoter region of the gymnosperm 4CL gene *involved in syringyl lignin biosynthesis.*

Voo discloses a 142 base pair sequence that is **not sufficient nor essential** for promoter expression. Exhibit A displays the location of the PstI site and the position of the start codon in

both the 4CL1B promoter sequence and the *Pinus radiata* 4CL promoter sequence. The data strongly indicate that the start of transcription is at least 135 base pairs upstream of the start codon in both sequences. Thus, the 135 base pair segment is a 5' untranslated region, which is not part of the functional promoter. Accordingly, the sequence disclosed in Voo is not part of the functional promoter claimed in the present application. Thus, Voo fails to anticipate the claimed invention. The rejection is therefore improper and should be withdrawn.

CONCLUSION

All of the stated grounds of rejections have been properly traversed or rendered moot. Therefore, the present application is now in condition for allowance, and an early notice to that effect is earnestly solicited.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. § 1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 12/19/06

By _____ 35,087 for

FOLEY & LARDNER LLP
Customer Number: 22428
Telephone: (202) 672-5483
Facsimile: (202) 672-5399

Richard C. Peet
Attorney for Applicants
Registration No. 35,792

EXHIBIT A

Section 1

```
                    (1) 1         10        20        30        43
MTU4CLPromoter(patent) (1) GGCCGGGTGGTGACATTTATTCATAAATTCATCTCAAAACAAG
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus              (1)
```

Section 2

```
                   (44) 44        50        60        70        86
MTU4CLPromoter(patent) (44) AAGGATTTACAAAAATAAAAGAAAACAAAATTTTCATCTTTAA
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus              (44)
```

Section 3

```
                   (87) 87        100       110       129
MTU4CLPromoter(patent) (87) CATAATTATAATTGTGTTCACAAAATTCAAACTTAAACCCTTA
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus              (87)
```

Section 4

```
                  (130) 130       140       150       160       172
MTU4CLPromoter(patent) (130) ATATAAAGAATTTCTTTCAACAATACACTTTAATCACAACTTC
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus             (130)
```

Section 5

```
                  (173) 173       180       190       200       215
MTU4CLPromoter(patent) (173) TTCAATCACAACCTCCTCCAACAAAATTAAAATAGATTAATAA
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus             (173)
```

Section 6

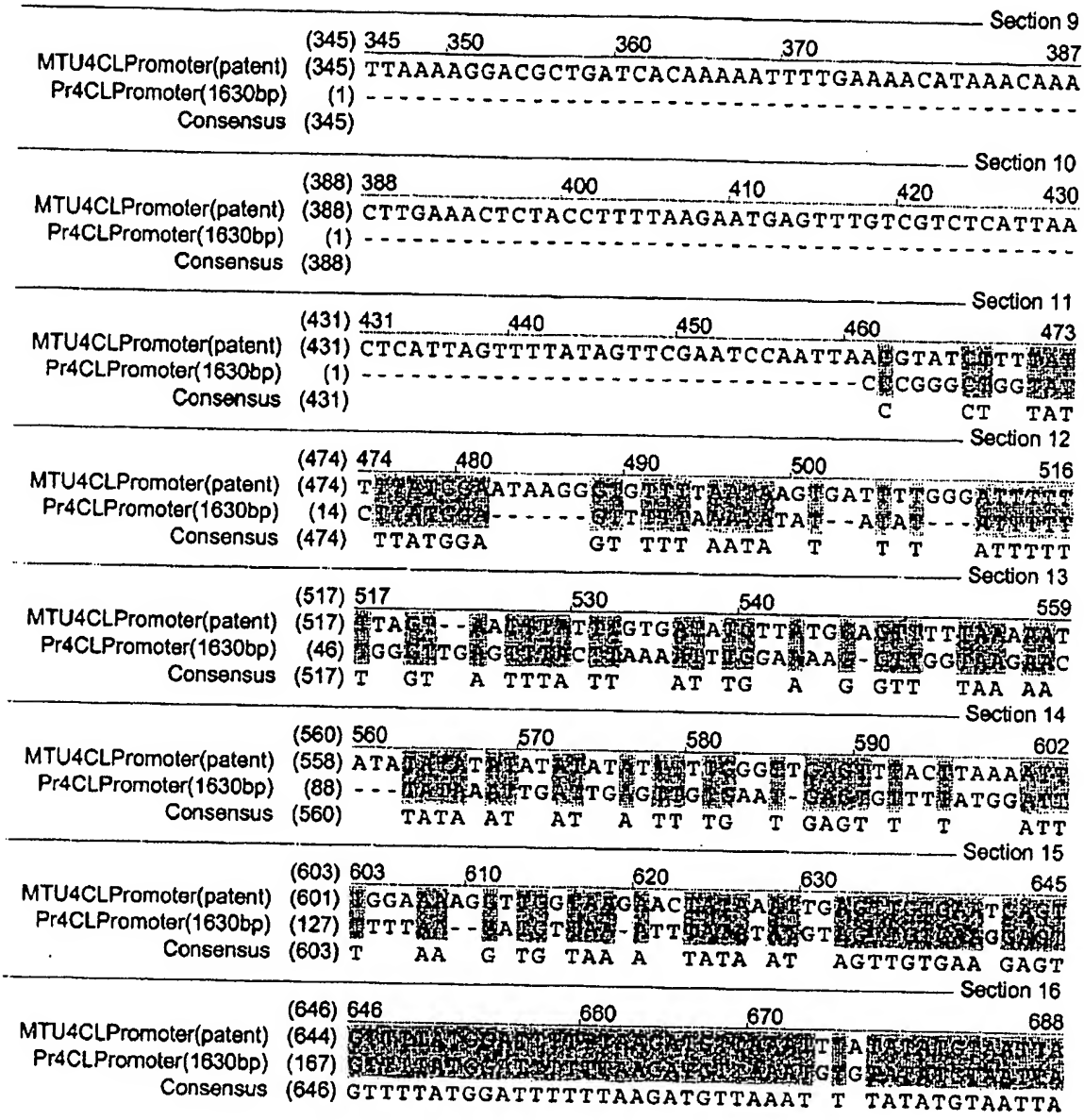
```
                  (216) 216       230       240       258
MTU4CLPromoter(patent) (216) ATAAATAAACTTAACTATTTAAAAAAAAATATTATACAAAATT
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus             (216)
```

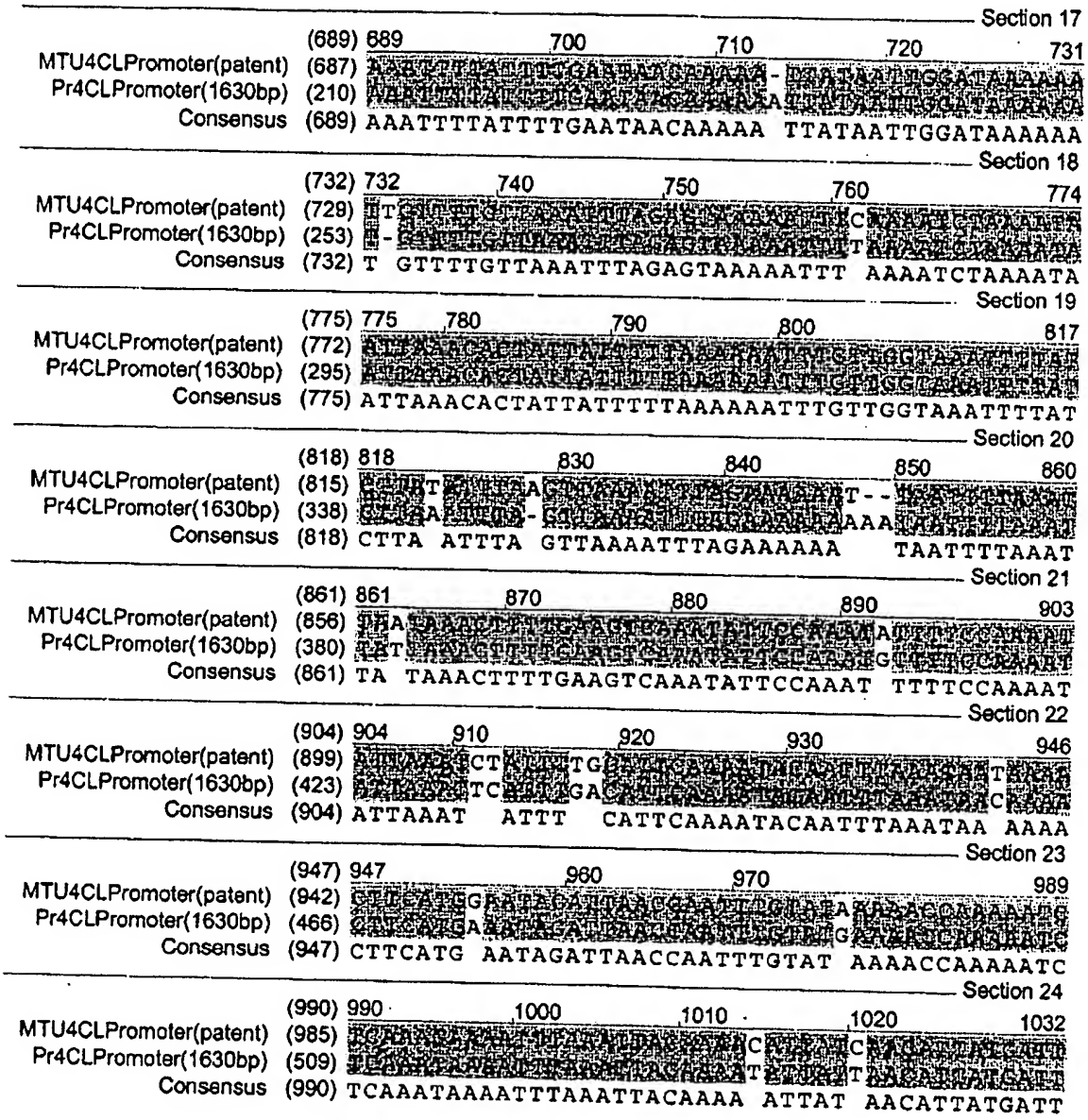
Section 7

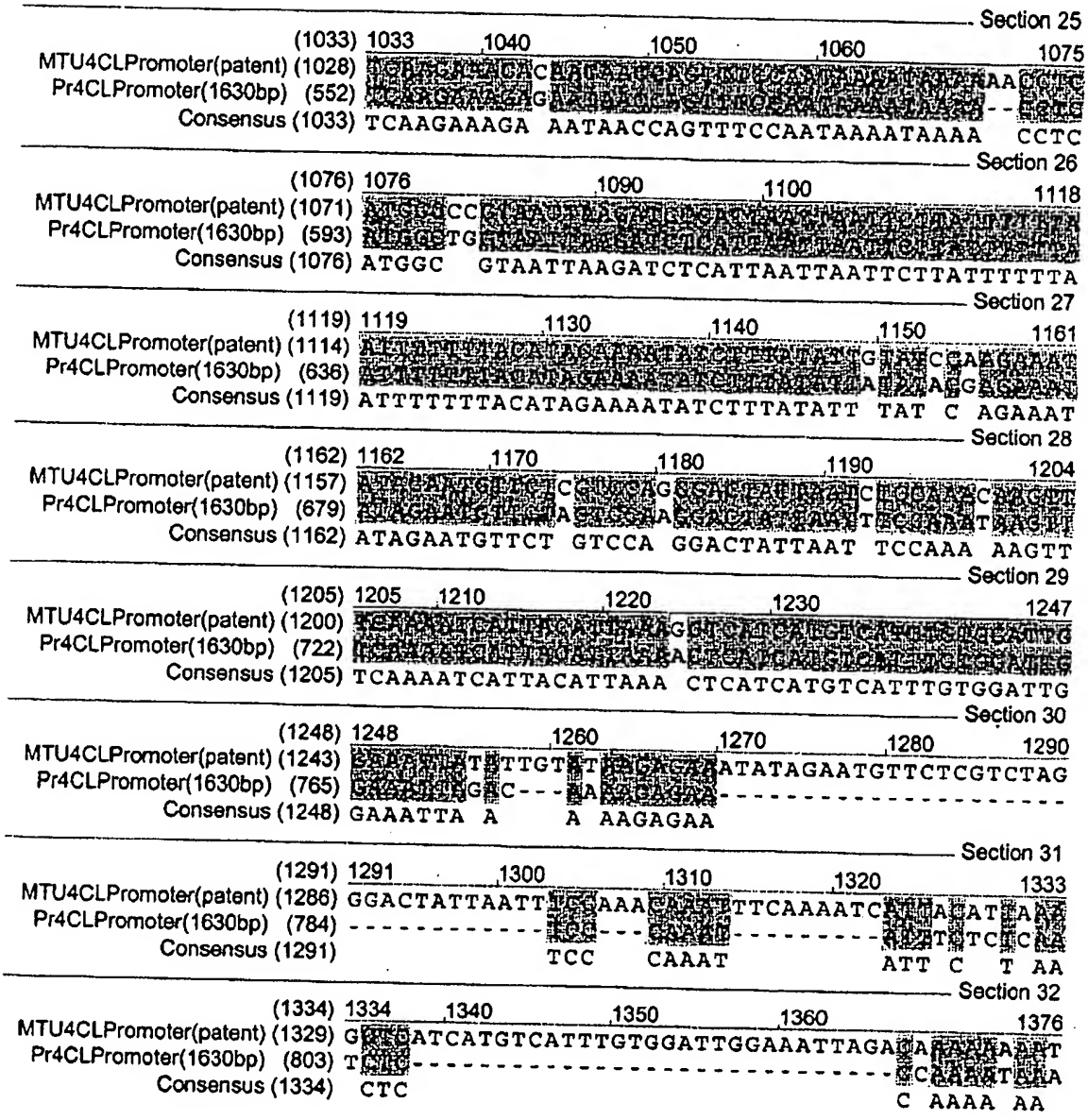
```
                  (259) 259       270       280       290       301
MTU4CLPromoter(patent) (259) TATTAAAACTTCAAAATAAACAAACTTTTTATACAAAATTCAT
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus             (259)
```

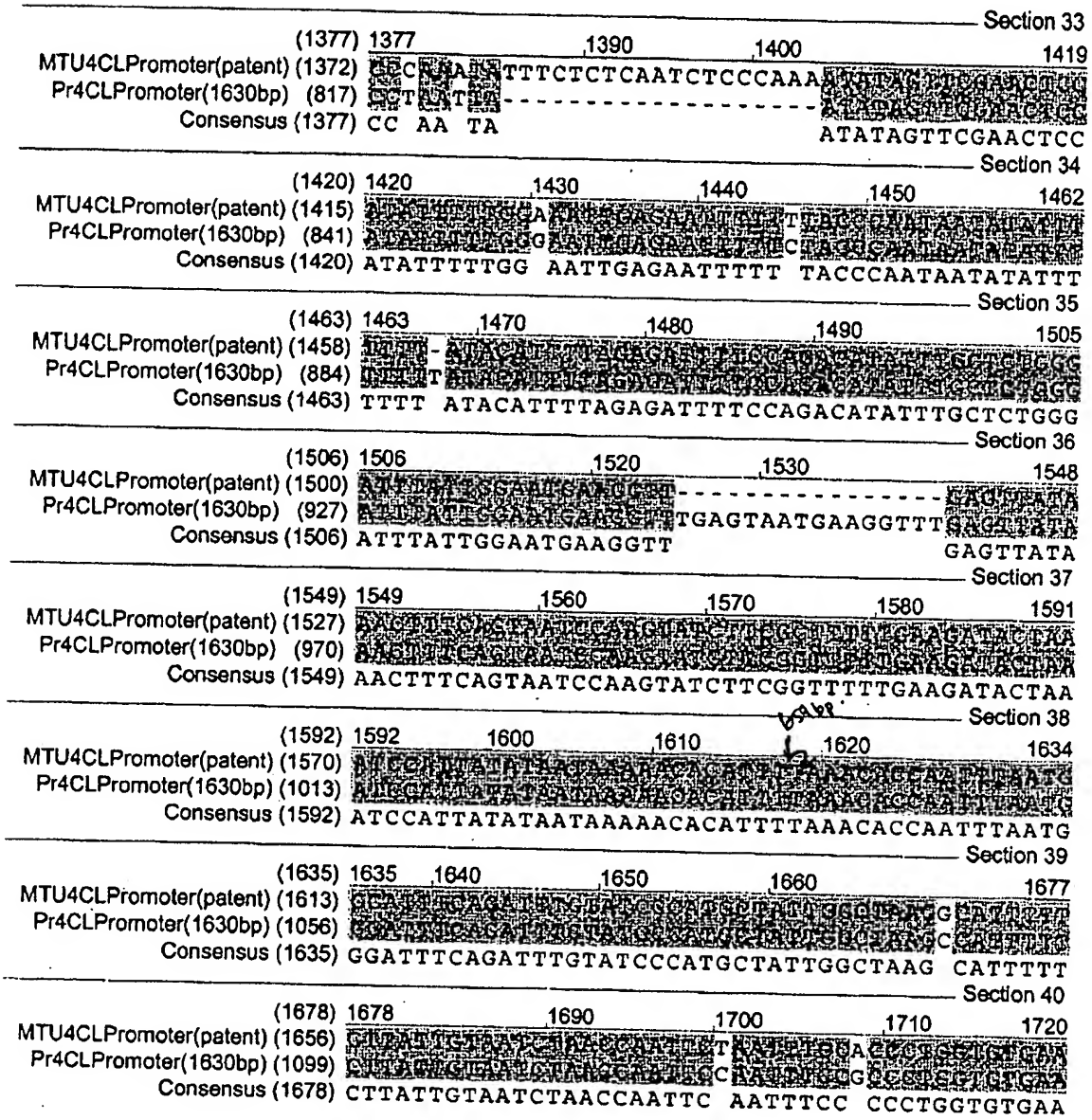
Section 8

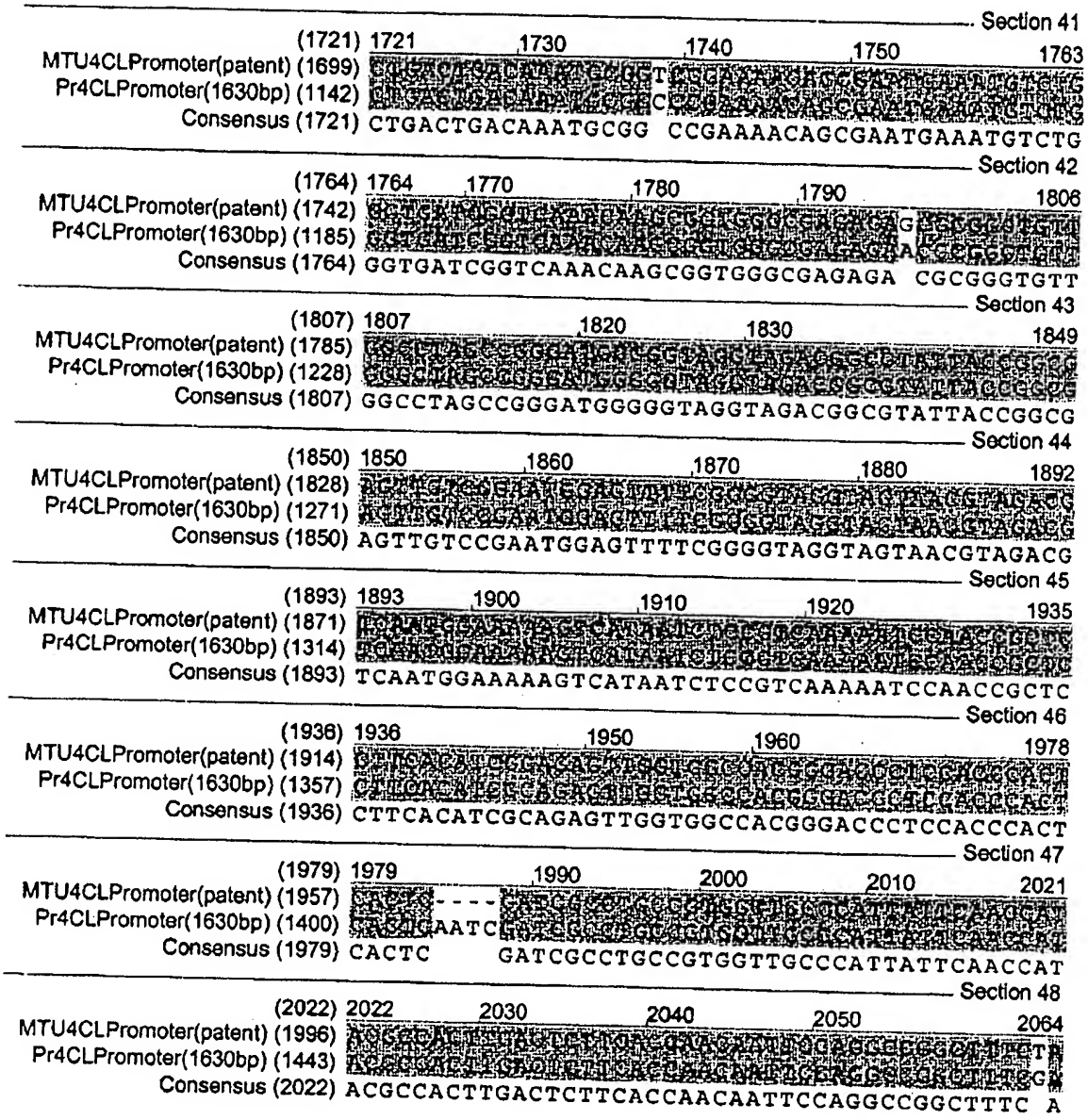
```
                  (302) 302       310       320       330       344
MTU4CLPromoter(patent) (302) CAAAACTTTAAAATAAAGCTAAACACTGAAAATGTGAGTACAT
Pr4CLPromoter(1630bp)  (1) -------------------------------------------
Consensus             (302)
```


Section 9
(345) 345 350 360 370 387
MTU4CLPromoter(patent) (345) TTAAAAGGACGCTGATCACAAAAATTTTGAAAACATAAACAAA
Pr4CLPromoter(1630bp) (1) ----------
Consensus (345)
Section 10
(388) 388 400 410 420 430
MTU4CLPromoter(patent) (388) CTTGAAACTCTACCTTTTAAGAATGAGTTTGTCGTCTCATTAA
Pr4CLPromoter(1630bp) (1) ----------
Consensus (388)
Section 11
(431) 431 440 450 460 473
MTU4CLPromoter(patent) (431) CTCATTAGTTTTATAGTTCGAATCCAATTAA
Pr4CLPromoter(1630bp) (1) ----------
Consensus (431)
Section 12
(474) 474 480 490 500 516
MTU4CLPromoter(patent) (474)
Pr4CLPromoter(1630bp) (14)
Consensus (474) TTATGGA GT TTT AATA T T T ATTTTT
Section 13
(517) 517 530 540 559
MTU4CLPromoter(patent) (517)
Pr4CLPromoter(1630bp) (46)
Consensus (517) T GT A TTTA TT AT TG A G GTT TAA AA
Section 14
(560) 560 570 580 590 602
MTU4CLPromoter(patent) (558)
Pr4CLPromoter(1630bp) (88)
Consensus (560) TATA AT AT A TT TG T GAGT T T ATT
Section 15
(603) 603 610 620 630 645
MTU4CLPromoter(patent) (601)
Pr4CLPromoter(1630bp) (127)
Consensus (603) T AA G TG TAA A TATA AT AGTTGTGAA GAGT
Section 16
(646) 646 660 670 688
MTU4CLPromoter(patent) (644)
Pr4CLPromoter(1630bp) (167)
Consensus (646) GTTTTATGGATTTTTTAAGATGTTAAAT T TATATGTAATTA


Section 17
(689) 689 700 710 720 731
MTU4CLPromoter(patent) (687)
Pr4CLPromoter(1630bp) (210)
Consensus (689) AAATTTTATTTTGAATAACAAAAA TTATAATTGGATAAAAAA
Section 18
(732) 732 740 750 760 774
MTU4CLPromoter(patent) (729)
Pr4CLPromoter(1630bp) (253)
Consensus (732) T GTTTTGTTAAATTTAGAGTAAAAATTT AAAATCTAAAATA
Section 19
(775) 775 780 790 800 817
MTU4CLPromoter(patent) (772)
Pr4CLPromoter(1630bp) (295)
Consensus (775) ATTAAACACTATTATTTTTAAAAAATTTGTTGGTAAATTTTAT
Section 20
(818) 818 830 840 850 860
MTU4CLPromoter(patent) (815)
Pr4CLPromoter(1630bp) (338)
Consensus (818) CTTA ATTTA GTTAAAATTTAGAAAAAA TAATTTTAAAT
Section 21
(861) 861 870 880 890 903
MTU4CLPromoter(patent) (856)
Pr4CLPromoter(1630bp) (380)
Consensus (861) TA TAAACTTTTGAAGTCAAATATTCCAAAT TTTTCCAAAAT
Section 22
(904) 904 910 920 930 946
MTU4CLPromoter(patent) (899)
Pr4CLPromoter(1630bp) (423)
Consensus (904) ATTAAAT ATTT CATTCAAAATACAATTTAAATAA AAAA
Section 23
(947) 947 960 970 989
MTU4CLPromoter(patent) (942)
Pr4CLPromoter(1630bp) (466)
Consensus (947) CTTCATG AATAGATTAACCAATTTGTAT AAAACCAAAAATC
Section 24
(990) 990 1000 1010 1020 1032
MTU4CLPromoter(patent) (985)
Pr4CLPromoter(1630bp) (509)
Consensus (990) TCAAATAAAATTTAAATTACAAAA ATTAT AACATTATGATT


Section 25
(1033) 1033 1040 1050 1060 1075
MTU4CLPromoter(patent) (1028)
Pr4CLPromoter(1630bp) (552)
Consensus (1033) TCAAGAAAGA AATAACCAGTTTCCAATAAAATAAAA CCTC
Section 26
(1076) 1076 1090 1100 1118
MTU4CLPromoter(patent) (1071)
Pr4CLPromoter(1630bp) (593)
Consensus (1076) ATGGC GTAATTAAGATCTCATTAATTAATTCTTATTTTTA
Section 27
(1119) 1119 1130 1140 1150 1161
MTU4CLPromoter(patent) (1114)
Pr4CLPromoter(1630bp) (636)
Consensus (1119) ATTTTTTTACATAGAAAATATCTTTATATT TAT C AGAAAT
Section 28
(1162) 1162 1170 1180 1190 1204
MTU4CLPromoter(patent) (1157)
Pr4CLPromoter(1630bp) (679)
Consensus (1162) ATAGAATGTTCT GTCCA GGACTATTAAT TCCAAA AAGTT
Section 29
(1205) 1205 1210 1220 1230 1247
MTU4CLPromoter(patent) (1200)
Pr4CLPromoter(1630bp) (722)
Consensus (1205) TCAAAATCATTACATTAAA CTCATCATGTCATTTGTGGATTG
Section 30
(1248) 1248 1260 1270 1280 1290
MTU4CLPromoter(patent) (1243)
Pr4CLPromoter(1630bp) (765)
Consensus (1248) GAAATTA A A AAGAGAA
Section 31
(1291) 1291 1300 1310 1320 1333
MTU4CLPromoter(patent) (1286) GGACTATTAATTTCGAAACAAATTTCAAAATCATTACATTAAA
Pr4CLPromoter(1630bp) (784)
Consensus (1291) TCC CAAAT ATT C T AA
Section 32
(1334) 1334 1340 1350 1360 1376
MTU4CLPromoter(patent) (1329) GTCATCATGTCATTTGTGGATTGGAAATTAGATAAAAAAATT
Pr4CLPromoter(1630bp) (803) TCTC
Consensus (1334) CTC C AAAA AA


Section 33
(1377) 1377 1390 1400 1419
MTU4CLPromoter(patent) (1372)
Pr4CLPromoter(1630bp) (817)
Consensus (1377) CC AA TA
ATATAGTTCGAACTCC
Section 34
(1420) 1420 1430 1440 1450 1462
MTU4CLPromoter(patent) (1415)
Pr4CLPromoter(1630bp) (841)
Consensus (1420) ATATTTTTGG AATTGAGAATTTTT TACCCAATAATATATTT
Section 35
(1463) 1463 1470 1480 1490 1505
MTU4CLPromoter(patent) (1458)
Pr4CLPromoter(1630bp) (884)
Consensus (1463) TTTT ATACATTTTAGAGATTTTCCAGACATATTTGCTCTGGG
Section 36
(1506) 1506 1520 1530 1548
MTU4CLPromoter(patent) (1500)
Pr4CLPromoter(1630bp) (927)
Consensus (1506) ATTTATTGGAATGAAGGTT GAGTTATA
Section 37
(1549) 1549 1560 1570 1580 1591
MTU4CLPromoter(patent) (1527)
Pr4CLPromoter(1630bp) (970)
Consensus (1549) AACTTTCAGTAATCCAAGTATCTTCGGTTTTTGAAGATACTAA
Section 38
(1592) 1592 1600 1610 1620 1634
MTU4CLPromoter(patent) (1570)
Pr4CLPromoter(1630bp) (1013)
Consensus (1592) ATCCATTATATAATAAAAACACATTTTAAACACCAATTTAATG
Section 39
(1635) 1635 1640 1650 1660 1677
MTU4CLPromoter(patent) (1613)
Pr4CLPromoter(1630bp) (1056)
Consensus (1635) GGATTTCAGATTTGTATCCCATGCTATTGGCTAAG CATTTTT
Section 40
(1678) 1678 1690 1700 1710 1720
MTU4CLPromoter(patent) (1656)
Pr4CLPromoter(1630bp) (1099)
Consensus (1678) CTTATTGTAATCTAACCAATTC AATTTCC CCCTGGTGTGAA


Section 41
(1721) 1721 1730 1740 1750 1763
MTU4CLPromoter(patent) (1699)
Pr4CLPromoter(1630bp) (1142)
Consensus (1721) CTGACTGACAAATGCGG CCGAAAACAGCGAATGAAATGTCTG
Section 42
(1764) 1764 1770 1780 1790 1806
MTU4CLPromoter(patent) (1742)
Pr4CLPromoter(1630bp) (1185)
Consensus (1764) GGTGATCGGTCAAACAAGCGGTGGGCGAGAGA CGCGGGTGTT
Section 43
(1807) 1807 1820 1830 1849
MTU4CLPromoter(patent) (1785)
Pr4CLPromoter(1630bp) (1228)
Consensus (1807) GGCCTAGCCGGGATGGGGGTAGGTAGACGGCGTATTACCGGCG
Section 44
(1850) 1850 1860 1870 1880 1892
MTU4CLPromoter(patent) (1828)
Pr4CLPromoter(1630bp) (1271)
Consensus (1850) AGTTGTCCGAATGGAGTTTTCGGGGTAGGTAGTAACGTAGACG
Section 45
(1893) 1893 1900 1910 1920 1935
MTU4CLPromoter(patent) (1871)
Pr4CLPromoter(1630bp) (1314)
Consensus (1893) TCAATGGAAAAAGTCATAATCTCCGTCAAAAATCCAACCGCTC
Section 46
(1936) 1936 1950 1960 1978
MTU4CLPromoter(patent) (1914)
Pr4CLPromoter(1630bp) (1357)
Consensus (1936) CTTCACATCGCAGAGTTGGTGGCCACGGGACCCTCCACCCACT
Section 47
(1979) 1979 1990 2000 2010 2021
MTU4CLPromoter(patent) (1957)
Pr4CLPromoter(1630bp) (1400)
Consensus (1979) CACTC GATCGCCTGCCGTGGTTGCCCATTATTCAACCAT
Section 48
(2022) 2022 2030 2040 2050 2064
MTU4CLPromoter(patent) (1996)
Pr4CLPromoter(1630bp) (1443)
Consensus (2022) ACGCCACTTGACTCTTCACCAACAATTCCAGGCCGGCTTTC A

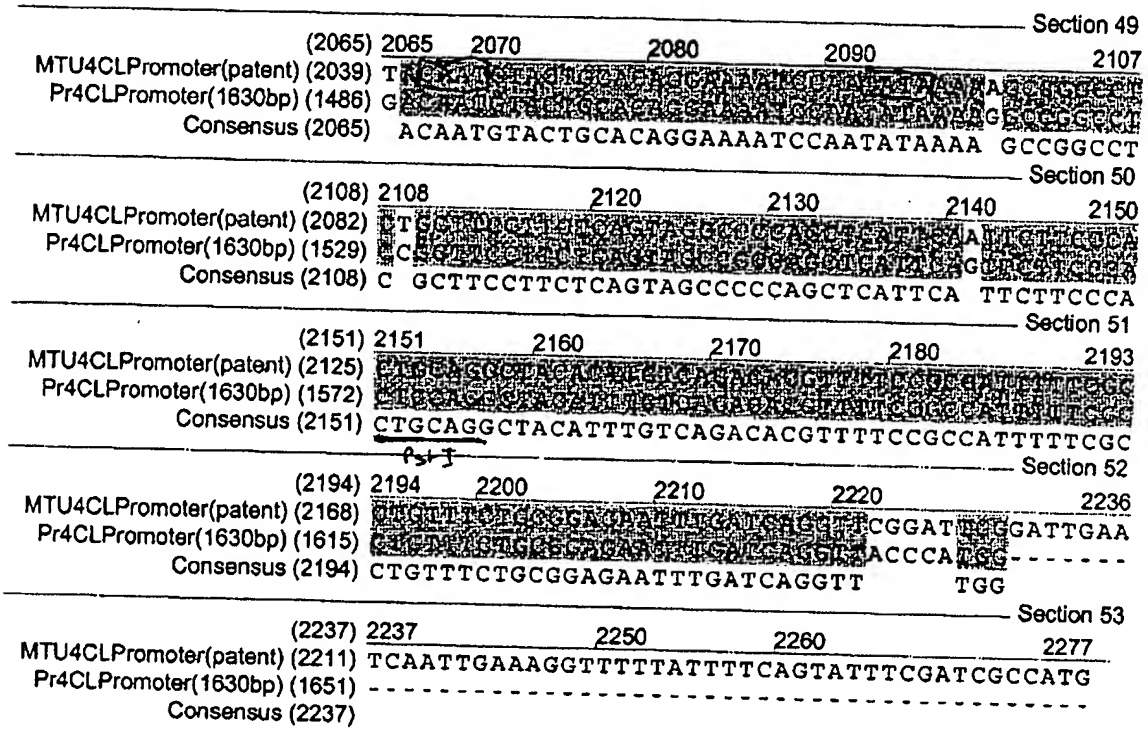

Section 49
(2065) 2065 2070 2080 2090 2107
MTU4CLPromoter(patent) (2039)
Pr4CLPromoter(1630bp) (1486)
Consensus (2065) ACAATGTACTGCACAGGAAAATCCAATATAAAA GCCGGCCT
Section 50
(2108) 2108 2120 2130 2140 2150
MTU4CLPromoter(patent) (2082)
Pr4CLPromoter(1630bp) (1529)
Consensus (2108) C GCTTCCTTCTCAGTAGCCCCCAGCTCATTCA TTCTTCCCA
Section 51
(2151) 2151 2160 2170 2180 2193
MTU4CLPromoter(patent) (2125)
Pr4CLPromoter(1630bp) (1572)
Consensus (2151) CTGCAGGCTACATTTGTCAGACACGTTTTCCGCCATTTTTCGC
Psi I
Section 52
(2194) 2194 2200 2210 2220 2236
MTU4CLPromoter(patent) (2168)
Pr4CLPromoter(1630bp) (1615)
Consensus (2194) CTGTTTCTGCGGAGAATTTGATCAGGTT TGG
Section 53
(2237) 2237 2250 2260 2277
MTU4CLPromoter(patent) (2211) TCAATTGAAAGGTTTTTATTTTCAGTATTTCGATCGCCATG
Pr4CLPromoter(1630bp) (1651)
Consensus (2237)

EXHIBIT B

The Plant Journal (2005) **43**, 553–567 doi: 10.1111/j.1365-313X.2005.02480.x

*Eg*MYB2, a new transcriptional activator from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis

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†Both authors contributed equally to this work.

Summary

***Eg*MYB2, a member of a new subgroup of the R2R3 MYB family of transcription factors, was cloned from a library consisting of RNA from differentiating *Eucalyptus* xylem. *EgMYB2* maps to a unique locus on the *Eucalyptus grandis* linkage map and co-localizes with a quantitative trait locus (QTL) for lignin content. Recombinant *Eg*MYB2 protein was able to bind specifically the *cis*-regulatory regions of the promoters of two lignin biosynthetic genes, cinnamoyl-coenzyme A reductase (*CCR*) and cinnamyl alcohol dehydrogenase (*CAD*), which contain MYB consensus binding sites. *Eg*MYB2 was also able to regulate their transcription in both transient and stable expression assays. Transgenic tobacco plants over-expressing *EgMYB2* displayed phenotypic changes relative to wild-type plants, among which were a dramatic increase in secondary cell wall thickness, and an alteration of the lignin profiles. Transcript abundance of genes encoding enzymes specific to lignin biosynthesis was increased to varying extents according to the position of individual genes in the pathway, whereas core phenylpropanoid genes were not significantly affected. Together these results suggest a role for *Eg*MYB2 in the co-ordinated control of genes belonging to the monolignol-specific pathway, and therefore in the biosynthesis of lignin and the regulation of secondary cell wall formation.**

Keywords: MYB, transcription, xylem, lignin, secondary cell wall.

Introduction

Lignin is one of the major components of the secondary walls of xylem cells, allowing mechanical support and efficient conduction of water and solutes over long distances within the vascular system. In woody plant species, a large proportion of photosynthetically assimilated carbon is channelled to lignin synthesis and, as a consequence, lignified cell walls represent a major proportion of plant biomass and a huge reservoir of carbon stored within the polymers of lignocelluloses (Boudet *et al.*, 2003).

Lignin biosynthesis involves the phenylpropanoid pathway, which converts phenylalanine to *p*-coumaroyl coenzyme A (CoA), the precursor of a wide range of phenolic compounds. The enzymes involved in this short sequence are *L*-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) (Figure 1). The subsequent hydroxylation and methylation steps have recently been shown to occur at the level of hydroxycinnamic acid esters and their corresponding aldehydes and/or alcohols (Humphreys and Chapple, 2002). The most likely route for the production of monolignols probably involves enzymatic reactions catalysed by *p*-hydroxycinnamoyl CoA: quinate/shikimate *p*-hydroxycinnamoyl

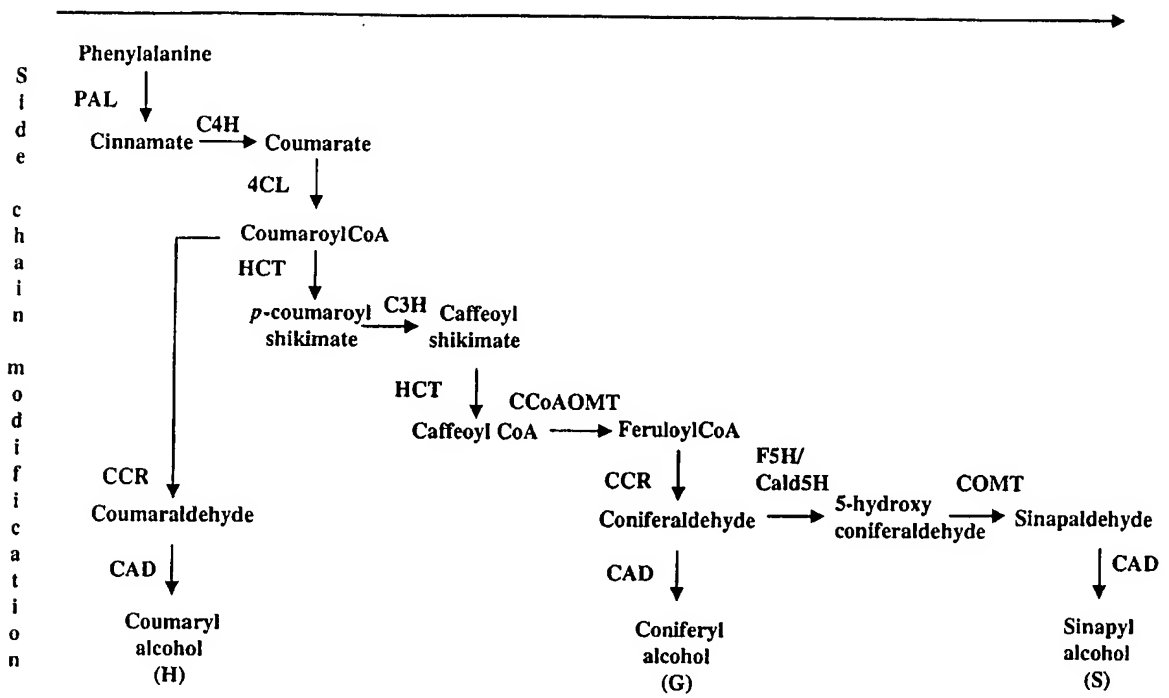


Figure 1. Model of monolignol biosynthesis pathway in angiosperms.
Enzymes involved in side-chain modification: PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate CoA ligase; HCT, *p*-hydroxycinnamoyl CoA: shikimate *p*-hydroxycinnamoyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase. SAD, sinapyl alcohol dehydrogenase. Enzymes involved in ring modification: C4H, cinnamate 4-hydroxylase; C3H, coumaroyl-quinate//shikimate 3-hydroxylase; CCoAOMT, caffeoyl CoA *O*-methyl transferase; F5H, ferulate 5-hydroxylase or Cald5H, coniferaldehyde-5-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase. The coumaryl, coniferyl and sinapyl alcohols are transported to the cell wall and polymerized to give rise to hydroxyphenyl (H), guaiacyl (G) and sinapyl (S) lignin, respectively.

transferase (HCT); coumaroyl-quinate/shikimate 3-hydroxylase (C3H); caffeoyl CoA *O*-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H, also called coniferaldehyde 5-hydroxylase, Cald5H); and caffeic acid *O*-methyltransferase (COMT) (for a review see Boerjan *et al.*, 2003) (Figure 1). Cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) catalyse the two last reductive steps leading to the three monolignols (*p*-coumaryl, coniferyl and syringyl alcohols), the monomeric units incorporated into the lignin heteropolymer.

In an attempt to gain insight into the mechanisms underlying the spatial and temporal control of lignification, we have characterized the genes encoding the terminal enzymes of lignin biosynthesis, CCR and CAD, and studied their expression during development. The promoters of the two genes from *Eucalyptus gunnii* (*EgCCR* and *EgCAD2*) direct expression in vascular tissues undergoing active lignification and preferentially in differentiating xylem (Feuillet *et al.*, 1995; Lacombe *et al.*, 2000; Lauvergeat *et al.*, 2002). The close correlation between *EgCCR* and *EgCAD2* promoter activities and lignification supports the view that tissue-specific transcription of these genes is a key step controlling the sites of lignin accumulation. Dissection of those promoters identified a *cis*-regulatory region of about 50–70 bp, responsible for driving this specific expression pattern in both promoters. The DNA–protein interaction sites have been mapped, and contain an AC-rich element corresponding to the MYB transcription factor binding consensus motif MBSIIG (MYB-binding site IIG, Lacombe *et al.*, 2000; Romero *et al.*, 1998; Sivadon and Grima-Pettenati, 2004).

The family of MYB transcription factors is one of the most abundant classes of transcription factors in plants, and the subfamily containing the two-repeat R2R3 DNA-binding domain is the largest (Stracke *et al.*, 2001). Some R2R3 MYB proteins bind AC elements found in the promoters of several genes of the phenylpropanoid pathway (Grotewold *et al.*, 1994; Sablowski *et al.*, 1994), and it has been suggested that these common motifs may provide a mechanism by which different steps of phenylpropanoid metabolism are co-ordinately regulated (Douglas, 1996; Martin and Paz-Ares, 1997; Weisshaar and Jenkins, 1998). Indeed, a number of R2R3 MYB proteins have been assigned functions in the regulation of phenylpropanoid biosynthesis and shown to regulate the biosynthesis of phenolic compounds, including lignin (Borevitz *et al.*, 2000; Jin *et al.*, 2000; Patzlaff *et al.*,

2003; Tamagnone *et al.*, 1998). Other transcription factors might also be involved (Rogers and Campbell, 2004).

As part of a programme aimed at characterizing 'lignin-specific' MYB *trans*-activators, we have cloned R2R3 MYB factors from a cDNA library of RNA from *Eucalyptus* differentiating xylem. Here we report the cloning and functional characterization of a new R2R3MYB gene, *EgMYB2*, which is able to bind the *EgCCR* and *EgCAD2* gene regulatory regions and to regulate their transcription. Transgenic tobacco plants over-expressing *EgMYB2* exhibited an increase in secondary wall thickness and an alteration in lignin composition. All the genes involved in the monolignol-specific biosynthesis pathway were upregulated, whereas the expression of core phenylpropanoid genes was not significantly affected. Taken together, the results reported here suggest that *Eg*MYB2 is a positive regulator of secondary cell wall formation and lignin biosynthesis.

Results

Eucalyptus Eg*MYB2 defines a new subgroup of the R2R3 MYB family*

To isolate MYB transcription factors potentially implicated in the regulation of lignin biosynthesis, we screened an *E. gunnii* xylem cDNA library (Lacombe *et al.*, 1997) with a MYB consensus sequence corresponding to the highly conserved amino acid sequence in the R3 repeat of the DNA-binding domain (Jackson *et al.*, 1991). Here we focus on the characterization of *EgMYB2* (AJ576023), a cDNA 1410 bp in length encoding a 321 amino acid protein (35.5 kDa, pI 4.92) which exhibits typical features of the R2R3 MYB protein family (Figure 2). The R2R3 DNA-binding domain comprises two imperfect repeats (54 and 51 amino acids, respectively), and has the predicted helix-turn-helix structures containing the conserved tryptophan residues involved in DNA binding (Martin and Paz-Ares, 1997). The predicted *Eg*MYB2 protein is closely related to other MYB proteins from different species within the R2R3 domain (Figure 2a,c) and shares the greatest homology with a *Populus trichocarpa* MYB protein *Poptr*1:49071 (86.4% similarity); and a MYB protein from Arabidopsis, *At*MYB83 (85.9% similarity; Romero *et al.*, 1998). Also closely related to *Eg*MYB2 are a MYB protein from a monocot species, *Hordeum vulgare* HvSPYMYB, *At*MYB46 and *Poptr*1:64485. A high level of homology was also found with a gymnosperm MYB protein, the pine *Pt*MYB4, which was recently shown to regulate lignin biosynthesis (Patzlaff *et al.*, 2003). *Eg*MYB2 belongs to group C as defined by Romero *et al.* (1998), most members of which bind preferentially to MBSIIG motifs G g/t T a/t GGT a/g.

The C-terminal domain of the *Eg*MYB2 protein does not contain any of the small conserved motifs used by Kranz *et al.* (1998) to classify MYB proteins in 22 subgroups. However, alignment of the C-terminal domain of *Eg*MYB2 with its closest related MYB proteins, *Poptr*1:49071 and *At*MYB83 (Figure 2b), revealed a conserved amino acid motif NX(R/K)(I/M)G(E/D)WDL(E/D)GL(M/I)(D/E)XXXSFPFLDF in the extreme C-terminal part of the protein. The presence of this C-terminal motif may define a new subgroup of MYB proteins, which possibly reflects the similar functions of its members.

EgMYB2 *maps to a unique locus on the* Eucalyptus grandis *linkage map and co-localizes with a QTL for lignin content*

The existence of a single *EgMYB2* gene in the *Eucalyptus* genome was supported by Southern hybridization at high stringency using the 3′ end as a probe (data not shown) and by genetic mapping. A full sib family of 201 interspecific hybrids between *Eucalyptus urophylla* and *E. grandis* was used to localize *EgMYB2* onto linkage maps previously constructed for both parents using RAPD markers (Verhaegen and Plomion, 1996; Verhaegen *et al.*, 1997). Using the single-strand conformation polymorphism (SSCP) technique (Orita *et al.*, 1989), *EgMYB2* was mapped on linkage group 2 of the *E. grandis* map (Figure 3). Interestingly, *EgMYB2* co-localizes with a quantitative trait locus (QTL) accounting for 4.5% of the variation in lignin content ($P = 0.009$). Although no linkage was found with the *E. urophylla* markers, a segregation (1:1:1:1) of the *EgMYB2* parental alleles was observed in the progeny. Using an ANOVA procedure between the four segregation classes, it was possible to discern a significant increase in *EgMYB2* maternal and paternal allele effects, explaining up to 7% of the variation of lignin content ($P = 0.005$).

EgMYB2 *is preferentially expressed in differentiating xylem tissue*

Using quantitative RT-PCR on *Eucalyptus* tissues, *EgMYB2* was shown to be preferentially expressed in the differentiating secondary xylem of stem and root, and in the central vein isolated from mature leaves (Figure 4). It is also expressed, albeit to a lesser extent, in the young part of stems and in young leaves containing developing veins. *EgMYB2* transcripts were present at very low levels in stem phloem, mature leaf blades, root bark and young lateral roots. This expression profile in lignin-rich tissue, which is in agreement with the fact that *Eg*MYB2 had been cloned from a *Eucalyptus* xylem cDNA library, could suggest that *Eg*MYB2 is involved in the regulation of lignin biosynthesis. Therefore we tested the effects of *Eg*MYB2 on two possible targets, the specific monolignol biosynthetic genes *EgCCR* and *EgCAD2*, which were also shown to be highly and preferentially expressed in *Eucalyptus* differentiating xylem (Grima-Pettenati *et al.*, 1993; Lacombe *et al.*, 1997).

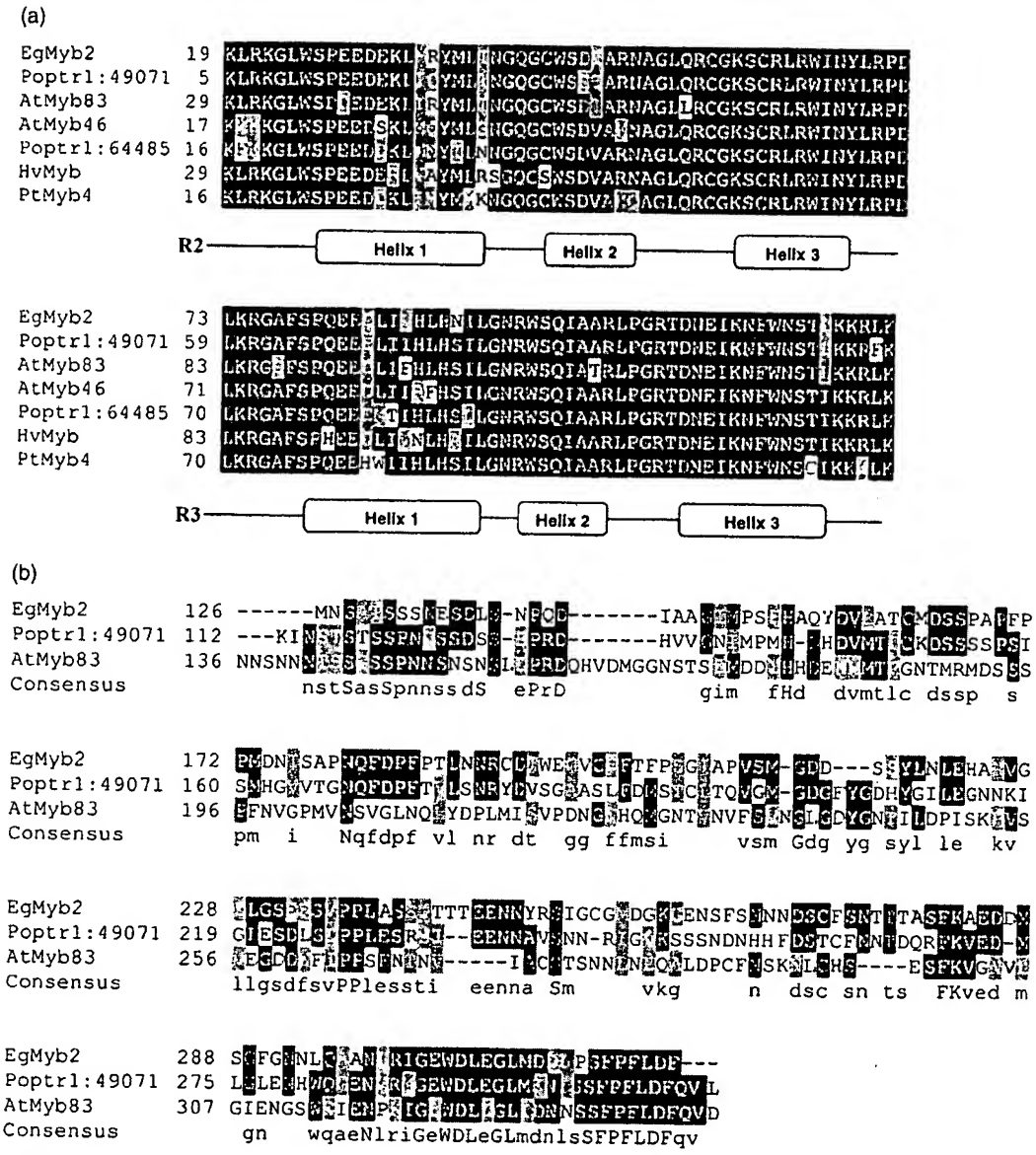


Figure 2. Sequence analysis of the *Eg*MYB2 protein.

(a) Amino-acid sequence alignment of the R2R3 domains of *Eg*MYB2 and other plant R2R3 MYB proteins. Multiple sequence alignments were generated using CLUSTALW (Thompson *et al.*, 1994). Residues highlighted in black are identical in more than 50% of the sequences; those highlighted in grey indicate conserved amino acid substitutions. The boxes below the alignment represent the predicted helix structure composing the two repeats.

(b) Alignment of the C-terminal regions of *Eg*MYB2 and its closest related MYB protein homologues. A conserved amino acid motif is remarkable in the extreme C-terminal region.

(c) Phylogenetic analysis of the R2R3 domain of *Eg*MYB2. Neighbour joining tree generated with MEGA 2.1 (Kumar *et al.*, 2001) using 1000 random sequence-addition bootstrap replication.

Genbank accession numbers: *Eg*MYB2 (AJ576023), *At*MYB46 (NM121290), *At*MYB83 (NM111685), *Pt*MYB4 (AY356371), *At*MYB4 (NM120023), *Hv*MYB3 (X70881), *Hv*SPYMYB (AY672068), *Le*MYB1 (X95297), *At*MYB61 (NP172425), *At*MYB55 (AF176000), *Dn*MYB2 (AF485893); *Poptr1*:49071 and *Poptr1*:64485 were found in the first draft of the *Populus trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html).

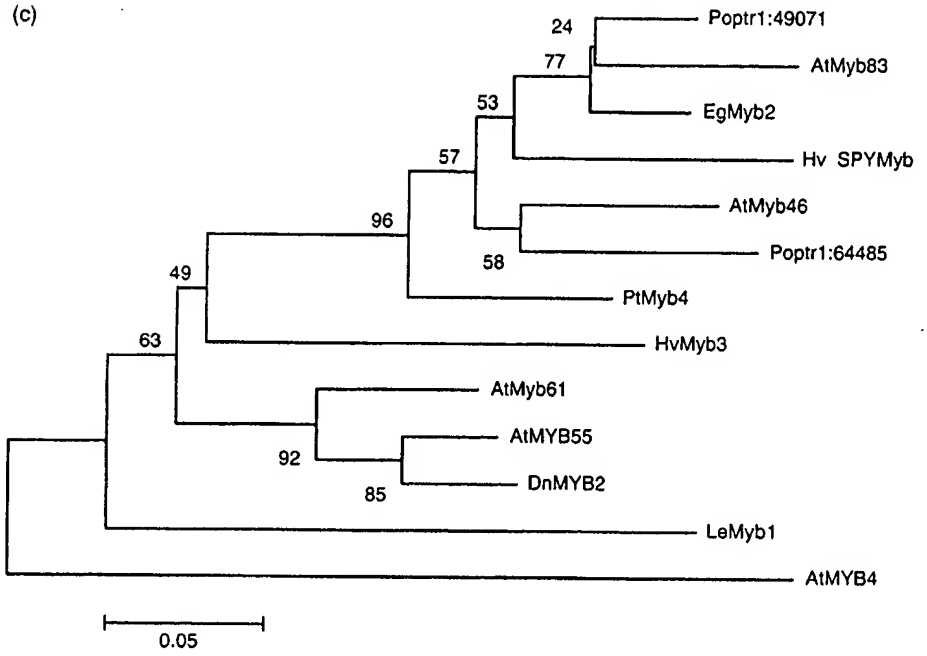


Figure 2. Continued.

Eg*MYB2 specifically binds the* cis*-regulatory regions of the* EgCCR *and* EgCAD2 *promoters*

As a first step to evaluate whether *Eg*MYB2 is involved in the transcriptional regulation of the *EgCCR* and *EgCAD2* genes, we tested the ability of *Eg*MYB2 to bind their *cis*-regulatory regions (Figure 5a) which contain MBSIIG sites (Figure 5b). For this purpose, the *EgMYB2* cDNA was fused to the glutathione-*S*-transferase coding sequence (*GST*) and expressed in *Escherichia coli* cells. Purified GST–*Eg*MYB2 protein was tested in electrophoretic mobility-shift assay (EMSA) for its ability to bind the cognate regulatory regions of *EgCAD2* (−203 to −129) and *EgCCR* (−119 to −70) promoters (Lacombe *et al.*, 2000; Lauvergeat *et al.*, 2002).

As shown in Figure 5(c), EMSA experiments revealed that *Eg*MYB2 is able to bind to both the *EgCAD2* (Figure 5c, lane 1) and *EgCCR* (Figure 5c, lane 5) promoter fragments. No DNA-binding activity was observed when using recombinant GST alone (data not shown), indicating that the interaction occurs specifically with EgMYB2. A 100-fold molar excess of unlabelled non-specific DNA fragment had no effect on the complex formation (Figure 5c, lanes 3 and 8), whereas effective competition was observed using either *EgCAD2* or *EgCCR* promoter fragments as specific competitors (Figure 5c, lanes 2 and 6). Cross-competition experiments have also been performed (Figure 5c, lanes 4 and 7), showing the ability of the *EgCAD2* and *EgCCR* promoter regions to compete reciprocally with the *Eg*MYB2 protein for binding. These gel-shift experiments show that recombinant *Eg*MYB2 is able to bind specifically *in vitro* to the regulatory regions of the *EgCCR* and *EgCAD2* promoters. These results raise the possibility that *Eg*MYB2 could control the co-ordinated expression of these two genes involved in the monolignol biosynthetic pathway.

Eg*MYB2 acts as a transcriptional activator of* EgCCR *and* EgCAD2 *promoters* in vivo

To test whether *Eg*MYB2 could transcriptionally regulate *EgCCR* and *EgCAD2* genes *in vivo*, the *EgCCR* and *EgCAD2* promoters fused to the GUS gene were used as reporter constructs (Lacombe *et al.*, 2000; Lauvergeat *et al.*, 2002) (Figure 6a). Each was co-transfected by *Agrobacterium* infiltration of *Nicotiana tabacum* (tobacco) leaves with an effector construct under the control of the 35S CaMV promoter, containing either the *EgMYB2* cDNA (referred to as *EgMyb2*$^+$), or the *EgMYB2* DNA-binding domain only (referred to as EgMyb2$^-$) (Figure 6a). Control values obtained using an effector construct without the *EgMYB2* gene (pJR1) exhibited significant levels of GUS activity. Interestingly, when *EgCCR* and *EgCAD2* promoters were co-transfected with *Eg*MYB2$^+$ construct, an induction of GUS activity was observed (Figure 6b). This increase was obtained reproducibly in several independent experiments, and was slightly higher for *EgCAD2* (2.4-fold) than for *EgCCR*

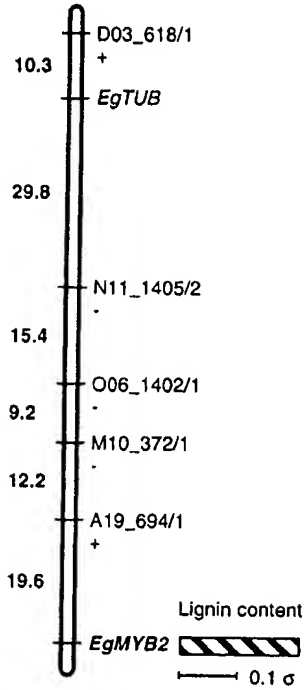


Figure 3. Genetic mapping of *EgMYB2* on *Eucalyptus* RAPD maps.
Using the single-strand conformation polymorphism (SSCP) technique, *EgMYB2* was located on *Eucalyptus grandis* linkage group 2 (for detailed maps see Verhaegen and Plomion, 1996). Distances along the linkage group are Kosambi centimorgans (cM); framework markers were ordered with an interval support ≥2. *EgMYB2* co-localizes with a QTL peak accounting for 4.5% of the phenotypic variation of the lignin content. *EgMYB2* effect is shown in cross-hatched bars expressed as phenotypic standard deviation (σ, difference between favourable QTL genotype and population mean).

(1.8-fold). Student's tests showed the relevance of these transactivation levels with significant values of $P < 0.001$ and $P < 0.04$ for transactivation of *EgCAD* and *EgCCR* promoters, respectively. The C-terminal region of *Eg*MYB2 appears to be the domain responsible for transcriptional activation of both promoters, as no activation was found using the *EgMYB2*$^-$ effector construct which contains only the DNA-binding domain.

Phenotypic changes induced by ectopic expression of Eg*MYB2 in transgenic tobacco*

To gain an insight into the role of *Eg*MYB2 *in planta*, we generated 18 independent transgenic tobacco plants expressing *EgMYB2* under the control of the 35S promoter (*EgMyb2*$^+$), and 15 dominant negative mutants expressing only the DNA-binding domain (*EgMyb2*$^-$).

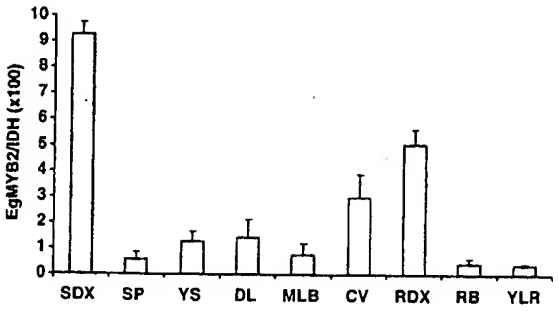


Figure 4. Expression of *EgMYB2* in *Eucalyptus* tissues.
Expression of *EgMYB2* was monitored by quantitative RT-PCR on RNA extracted from tissues of glasshouse-grown *Eucalyptus globulus*: stem differentiating secondary xylem, SDX; stem phloem, SP; young stems, YS; developing leaves, DL; mature leaf blades, MLB; central veins, CV; main root differentiating xylem, RDX; bark, RB; young lateral roots, YLR. Results are expressed as number of molecules (x100) relative to *Eg*IDH expression level as internal standard (see Experimental procedures). Two replicates were conducted using three independent biological individuals. Means and standard deviations are shown.

When grown *in vitro*, there were no obvious developmental differences among the primary transformants and control plants. After transfer to the glasshouse, *EgMyb2*$^-$ plants still showed no visible differences in growth and/or phenotypic aspect relative to control plants, whereas *EgMyb2*$^+$ plants exhibited a number of phenotypic differences (Figure 7). Half the plants transformed with the *EgMyb2*$^+$ construct (9/18) grew to only two-thirds of the size of the control plants (Figure 7a). Many of the *EgMyb2*$^+$ plants had two main stems emerging at the base of the plant (11/18) (Figure 7b), an effect generally associated with a loss of apical dominance. Interestingly, in hand-cut stem sections most plants also exhibited orange coloration of the xylem ring compared with the yellowish xylem of control plants (Figure 7c), probably reflecting an alteration of the secondary cell wall composition. Five independent primary transformants (2.7; 2.16; 2.18; 2.19; 2.24), all exhibiting orange-coloured xylem, were selected for study of segregation of the transgene in the progeny. Like most of the *EgMyb2*$^+$ plants they showed a significant reduction in seed production compared with controls, and the seeds were paler. Moreover, seeds of three transformants (2.18; 2.19; 2.24) were unable to germinate despite repeated attempts involving changes to the sterilization treatment, and even with no sterilization treatment at all. The percentage of germination of seeds from transformants 2.7 and 2.16 were 50 and 75%, respectively.

As a first step towards understanding why the seed did not germinate at all, or with a dramatically reduced efficiency, lignin staining was performed using phloroglucinol on seeds from control plants and transformants 2.7 and 2.18 (Figure 7d–f). Approximately half the seeds of transformant 2.7 stained more intensely than the control seeds, in

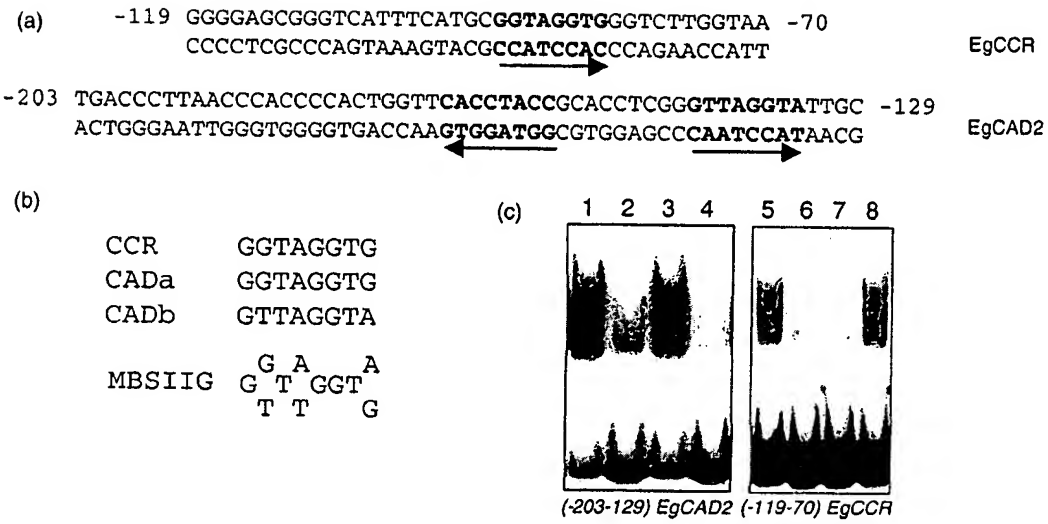
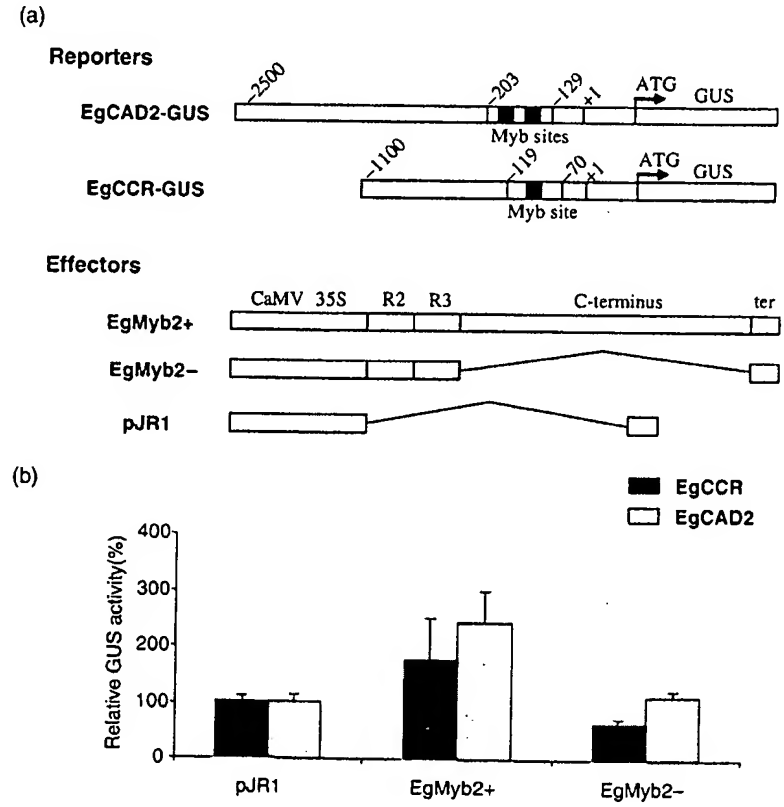


Figure 5. Binding of *Eg*MYB2 to the *cis*-regulatory regions of the *EgCAD2* and *EgCCR* promoters.
(a) Nucleotide sequences of *EgCAD2* and *EgCCR* promoter fragments used in electrophoretic mobility-shift assay (EMSA) experiments. These fragments correspond to the *cis*-regulatory regions involved in vascular expression. Positions given with respect to transcription start site. AC elements indicated in bold with arrows indicating their orientation.
(b) Nucleotide sequences of AC elements found in *EgCCR* and *EgCAD2* promoters compared with the MBSIIG consensus site (Romero *et al.*, 1998).
(c) EMSA in which the indicated radiolabelled promoter fragments were incubated with GST–*Eg*MYB2 recombinant protein in the absence (lanes 1, 5) or presence of 100× molar excess of non-specific (lanes 3, 8), [−203–129] *EgCAD2* (lanes 2, 7) or [−119–70] *EgCCR* (lanes 4, 6) unlabelled competitors. Amounts of GST–*Eg*MYB2 protein were 100 and 30 ng for *EgCAD2* and *EgCCR* promoter fragments, respectively. No DNA-binding activity was observed using recombinant GST alone (data not shown).

Figure 6. Effects of *Eg*MYB2 on transcriptional activities of the *EgCAD2* and *EgCCR* promoters *in vivo*.
(a) Schematic maps of the reporter and effector constructs. CaMV35S, 35S promoter; GUS, *uidA* coding region; (+1), transcription start site; R2R3, MYB DNA-binding domain; ter, nopaline synthase terminator.
(b) Results from co-transfection experiments in tobacco leaves. Agrobacteria containing effector and reporter constructs were co-infiltrated in tobacco leaves. Data represent mean values and standard deviations of three independent experiments, each containing at least three replicates. GUS activity is expressed as percentage of GUS activity relative to control (pJR1, 'empty' vector co-transfected with reporter constructs). Activations of *EgCAD* and *EgCCR* promoters are statistically significant relative to controls (Student's $P < 0.001$ and 0.04, respectively).



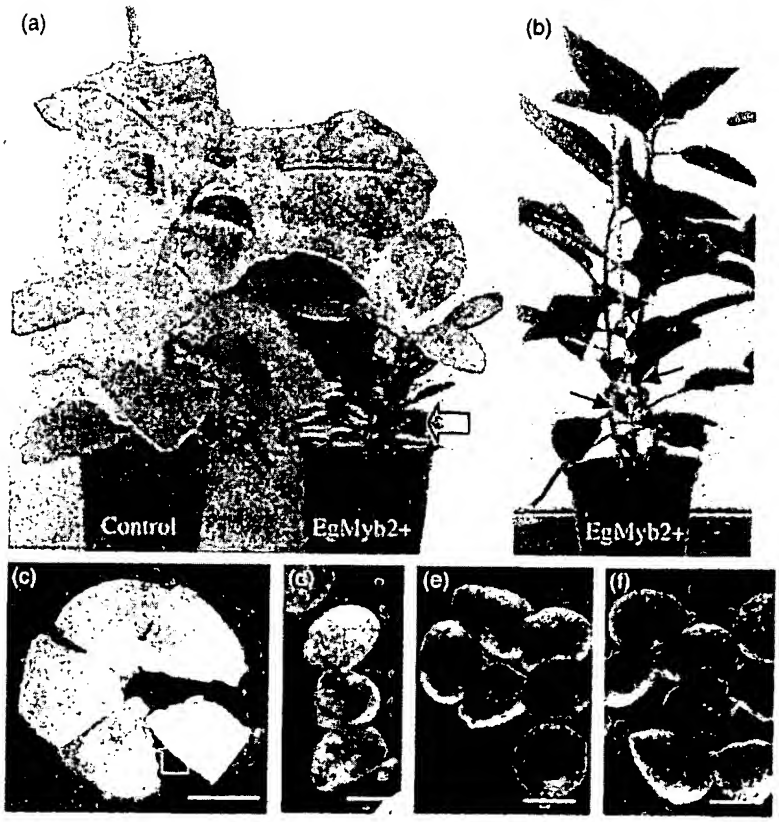


Figure 7. Phenotypic changes induced by *EgMYB2* ectopic expression in tobacco.
(a) Control plant and *EgMyb2*$^+$ primary transformant 2 weeks after transfer to the glasshouse.
(b) *EgMyb2*$^+$ primary transformant 6 weeks after transfer to the glasshouse. Note the presence of two main stems (arrows).
(c) Pattern of xylem coloration in hand-cut stem sections of four *EgMyb2*$^+$-independent primary transformants relative to control stem section (open arrow), scale 0.5 cm.
(d–f) Phloroglucinol staining of seeds (scale 0.8 mm): seeds of control (d); *EgMYB2*$^+$ primary transformant 2.7 (e); and transformant 2.18 (f).

agreement with the 50% germination rate (Figure 7e). All seeds of 2.18 which were unable to germinate stained more intensely than control seeds. Staining was concentrated in the region of the micropylar testa, where the radicle is known to emerge (Figure 7f).

The main phenotypic characteristics of primary transformants 2.7 and 2.16 (reduced plant size, loss of apical dominance, orange coloration of xylem) were associated with transgene expression, as these characteristics were maintained in the T_1 and T_2 progeny.

Eg*MYB2 increases xylem secondary cell wall thickness*

Cytological observations of xylem were performed on glasshouse-grown tobacco stem sections, either at low magnification using epifluorescence microscopy (Figure 8a,b) or at higher magnification using confocal microscopy (Figure 8c,d). At low magnification a significant increase in the number of lignified phloem fibres and xylem vessels was observed in some transformants, such as 2.18 (Figure 8b), compared with controls (Figure 8a).

Observations under confocal microscopy revealed that the xylem cell walls were thicker in the *Eg*MYB2$^+$ transformants (Figure 8d) compared with controls (Figure 8c). Measurements of fibre cell wall thickness indicate a dramatic thickening of xylem cell walls in transformant plants (4.9 ± 0.9 μm) compared with controls (3.5 ± 0.8 μm) (Figure 9). Student's test showed that the difference between the two populations is highly significant with $P < 0.001$ and $n = 500$. Observations of cell walls at the ultrastructural level by electron microscopy allowed us to demonstrate that the increase in cell wall thickness in *EgMyb2*$^+$ plants was due to an increase in the thickness of the middle S2 layer of the secondary cell wall (compare Figure 8g,h with controls in Figure 8e,f), whereas the S1 external and S3 internal layers appeared normal.

To gain information on the lignin content and composition within the xylem tissues, staining was performed using phloroglucinol reagent (Figure 8i,j); Maüle reagent (Figure 8k,l); and potassium permanganate for the visualization of lignin distribution (Figure 8m,n). The intensity of staining was always higher in transformed plants, whichever reagent was used, and appeared uniform across the thickened cell wall. The increase in reactivity of the cell walls using phloroglucinol and potassium permanganate could indicate a higher lignin content in xylem, which could be related to increased cell wall thickness. On the other hand, the strong reactivity to the Maüle reagent, which primarily stains syringyl lignin indicative of S units, suggests a higher S-unit composition in the lignin from transgenic lines.

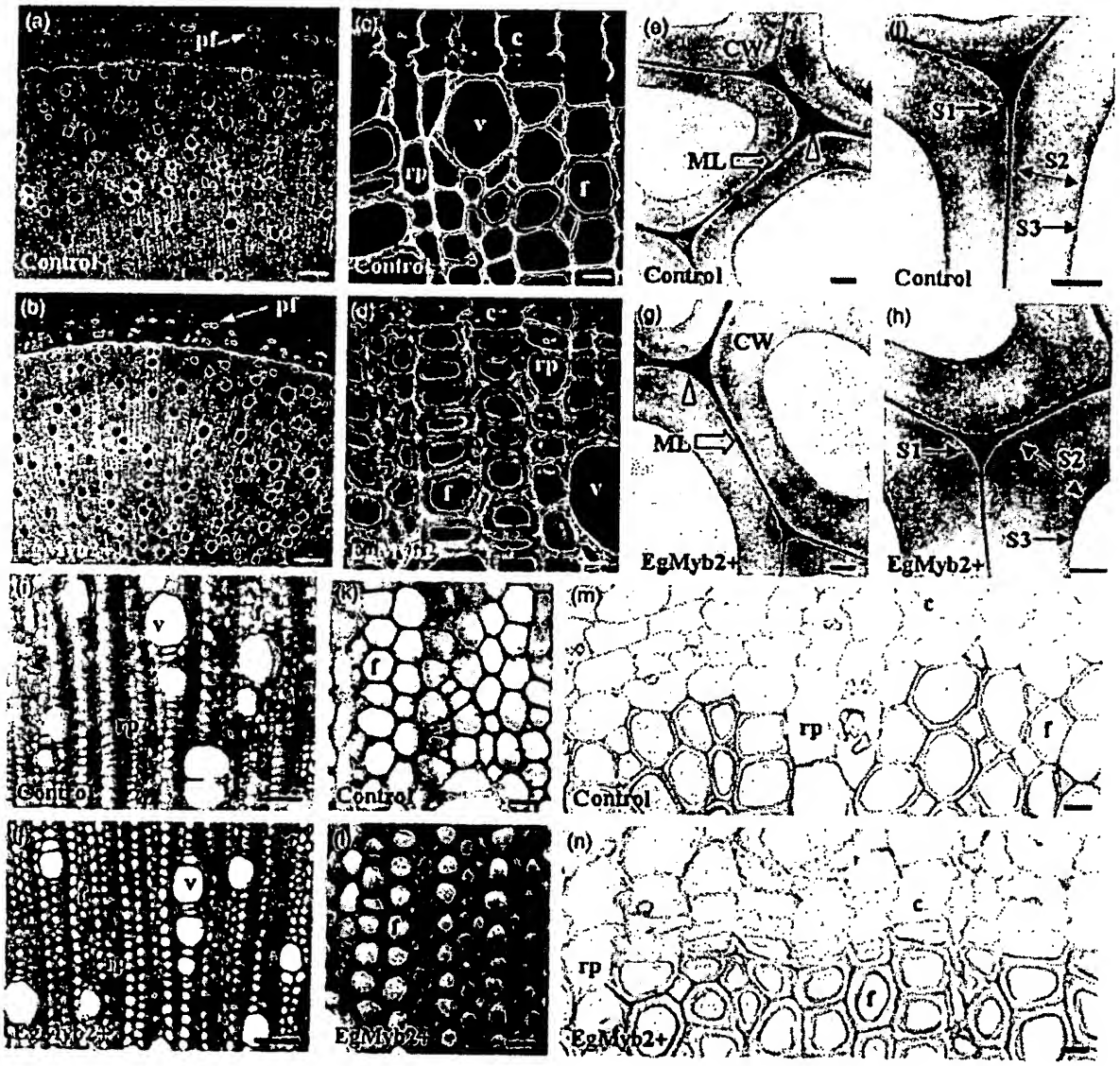


Figure 8. Cytological effects of *EgMYB2* over-expression in stem sections.
(a–d) Autofluorescence of xylem tissues on hand-cut stem sections under epifluorescence microscopy (a,b) and confocal microscopy (c,d) for control plant (a,c) and EgMYB2$^+$ transformant (b,d).
(e–h) Electron micrographs of $KMnO_4$-stained ultrathin stem sections of control (e,f) and *EgMyb2*$^+$ transformant (g,h).
(i–n) Phloroglucinol (i, j), Maüle (k,l) and potassium permanganate (m,n) stainings on transverse sections of control plants (i,k,m) and *EgMyb2*$^+$ transformant (j,l,n).
v, xylem vessel; rp, ray parenchyma; f, xylem fibres; pf, phloem fibres; c, cambial zone. S1–S3, layers within xylem fibre cell wall. Open arrows, middle lamella area (ML); arrowheads, cell junctions. Scale bars, 100 (a,b); 50 (i,j), 30 (k,l); 20 (c,d,m,n); 1 μm (e,f,g,h).

Eg*MYB2 controls the co-ordinated expression of genes involved in the lignin biosynthetic pathway*

The ability of *Eg*MYB2 to alter the expression of a complete set of genes involved in lignin biosynthesis (*PAL, C4H, 4CL, C3H, HCT, CCoAOMT, F5H, COMT, CCR, CAD*) was assessed by quantitative RT-PCR using RNA isolated from leaves of the transgenic lines described above. The assays were normalized to actin transcript levels. Figure 10 shows the relative levels of transcript accumulation found in glasshouse-grown *EgMyb2*$^+$ plants relative to control plants. Transcription of the two genes involved in the early steps of phenylpropanoid metabolism, *PAL* and *C4H*, was not significantly affected by the over-expression of *EgMYB2* and the third gene involved, *4CL*, appeared moderately (about threefold) activated. In marked contrast, all the genes

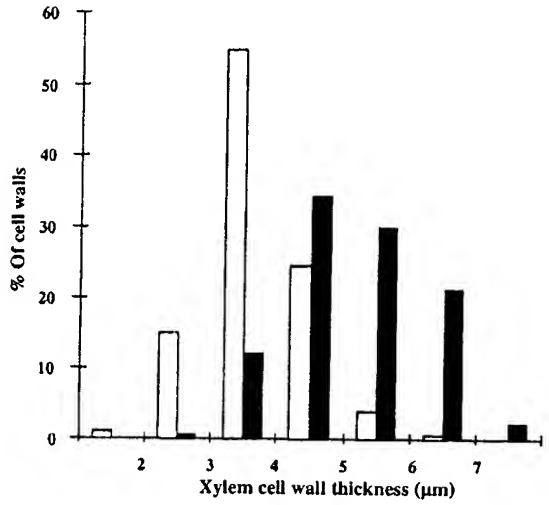


Figure 9. Effect of *EgMYB2* ectopic expression on the thickness of xylem fibres cell walls.
Frequency graph for comparison of cell wall thickness distribution (percentage of cell walls) between controls (open bars) and *EgMyb2*$^+$ plants (solid bars) within different classes. [0–2], thickness <2 µm; [2–3], thickness 2–3 µm, etc. 500 measurements were performed on semi-thin stem sections observed under bright light from two control plants and four independent primary transformants, using IMAGE-PRO PLUS software. Means and standard deviations, 4.9 ± 0.9 µm for *EgMyb2+* plants; 3.5 ± 0.8 µm for controls. The difference between the two populations is highly significant (Student's $P < 0.001$).

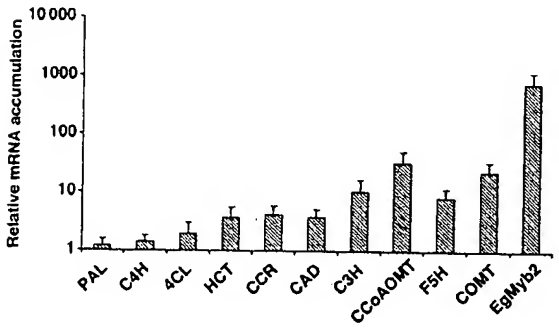


Figure 10. Quantitative RT-PCR analysis of transcript accumulation in transgenic tobacco plants over-expressing *EgMYB2*.
PAL, C4H, 4CL, C3H, HCT, COMT, F5H, CCoAOMT, CCR, CAD and *EgMYB2* mRNA accumulation was assessed by quantitative RT-PCR in leaves of four independent primary transformants. Transcript levels were normalized relative to the actin expression level as internal standard. Results expressed as mean and standard deviation relative to control plant the expression level of which has been assigned the value = 1 on the logarithmic scale.

encoding steps committed to monolignol biosynthesis were dramatically upregulated. Transcripts of the genes involved in conversion of esters to aldehydes, and aldehydes to alcohols (*HCT, CCR, CAD*) were about fivefold more abundant in transgenic *EgMyb2*$^+$ plants than in control plants, and transcript levels of the genes involved in the differential pathways leading to the three monolignol monomers (*C3H, CCoAOMT, F5H, COMT*) were up to 40-fold higher. Upregulation of the monolignol biosynthesis genes was also observed in stem tissue of the transgenic plants, but the extent was about twofold lower than in leaves, probably due to the fact that these genes are already activated by the endogenous tobacco MYB protein in stems.

Eg*MYB2 mainly controls the monomeric composition of lignin*

To test the effect of *Eg*MYB2 on lignin content and composition, we performed biochemical lignin determinations on the selected mature primary transformants. Despite the cytological observations and gene expression analysis described above, the data presented in Table 1 reveal that the Klason lignin content of the transformants does not differ substantially from the control level.

Lignin structure was investigated by thioacidolysis, a method that allows extraction of the lignin units involved in β-O-4 bonds (Lapierre *et al.*, 1986). The total yield of G and S lignin-derived main monomers recovered from the cell wall residue and referred to the Klason lignin was found to be similar in the transformants and control series. This result shows that the content of lignin units involved in uncondensed β-O-4 bonds is not significantly affected by the transgene. However, the S/G ratio was found to be significantly higher in the lignin extracted from *EgMyb2*$^+$ plants (1.27 ± 0.11 relative to control 1.09 ± 0.01, with Student's $P < 0.05$), essentially due to an increase in the amount of S units (Table 1). This result is consistent with the higher Maüle staining of the xylem samples from transgenic plants relative to the controls (Figure 8k,l). Interestingly, it is also related to the expression levels of the genes encoding CCoAOMT and COMT, enzymes involved in the methylation steps leading to the G and S monomeric units. Indeed, the highest S/G ratio in lignin (1.46, 34% higher than in control plants) was found in the transgenic plant in which the *COMT/CCoAOMT* expression ratio was highest (2.08). Conversely, the lowest S/G ratio among the transformants (1.18) was found in the plant in which the *COMT/CCoAOMT* expression ratio was lowest (0.16). The higher S/G value of the transformants relative to controls could be confirmed on T_2 plants (data not shown). Together, these data suggest that *Eg*MYB2 controls the *COMT/CCoAOMT* ratio which, in turn, has a direct consequence for the S/G monomeric ratio.

Discussion

As part of a programme aimed at identifying regulators of lignin biosynthesis, we have characterized a new R2R3 MYB

Table 1 Lignin analysis in *EgMyb2*$^+$ plants

Plant	Klason	S	G	S + G	S/G
2.7	20.91	919	749	1668	1.23
2.24	19.24	1019	867	1886	1.18
2.16	20.23	1070	854	1924	1.25
2.18	20.38	1077	881	1958	1.22
2.19	19.73	1163	798	1961	1.46
Mean *Eg*MYB2$^+$	20.10 ± 0.64	1049.6 ± 89.5	829.8 ± 55	1879 ± 122	1.27 ± 0.11
C06	19.02	953	874	1827	1.09
C30	19.7	1038	938	1976	1.1
C11	18.79	856	787	1643	1.09
Mean control	19.17 ± 0.47	949 ± 91	866.3 ± 75	1815 ± 166	1.09 ± 0.01

Five independent *Eg*MYB2$^+$ plants and three control plants were analysed for lignin content using the Klason method. Amount of lignin (referred to as Klason) is expressed as weight percentage of dried cell wall residue. Lignin composition was determined by thioacidolysis. Amounts of S and G monomers are expressed as μmol g^{-1} Klason lignin. Student's *t*-test showed that differences between controls and transgenics were significant with $P = 0.05$ for the S/G ratio. In contrast, the difference observed for the Klason lignin content does not appear as significant ($P = 0.07$).

gene (*EgMYB2*) from *Eucalyptus* that is highly and preferentially expressed in secondary xylem. This gene is single copy and maps to a QTL influencing lignin quantity. The *Eg*MYB2 protein is able to bind specifically the regulatory regions of the *EgCCR* and *EgCAD2* promoters *in vitro*, and to increase their transcription as shown by transient expression experiments. Together, these results raised the possibility that *Eg*MYB2 might control the co-ordinated expression of genes committed to the monolignol biosynthetic pathway. This hypothesis was further supported by transcript analysis of phenylpropanoid genes in transgenic tobacco plants over-expressing *EgMYB2*, which revealed significant increases in transcript abundance of genes known to be involved in the monolignol-specific portion of the pathway, but not those of the general phenylpropanoid pathway (*PAL*, *C4H*). The genes involved in conversion of esters to aldehydes, and of aldehydes to alcohols (side-chain modification), *HCT*, *CCR* and *CAD*, were about fivefold upregulated, whereas the genes involved in the differential pathways leading to the monolignol monomers (ring modification), *C3H*, *F5H*, *CCoAOMT* and *COMT*, were up to 40-fold upregulated.

As a consequence of the high increase in transcript abundance of the genes encoding enzymes involved in ring modification, the major main effect of *Eg*MYB2 was to alter the lignin monomeric composition. In angiosperms, the type of monomeric units within a lignin polymer depends on the degree of methylation of either the 3-hydroxyl groups or both 3-hydroxyl and 5-hydroxyl groups, leading to G and S units, respectively. CCoAOMT is believed to play an essential role in the synthesis of G units as well as in the supply of substrates for the synthesis of S units, whereas COMT essentially controls the biosynthesis of S units (Pincon *et al.*, 2001; Zhong *et al.*, 1998, 2000). In *EgMyb2*$^+$ plants we observed an increase in S/G ratio, mainly due to an increase in S units which probably results from the strong activation of *COMT*.

At the phenotypic level, plants over-expressing *Eg*MYB2 were characterized by reduced size in comparison with controls. With regard to this observation, it is interesting to draw a parallel with the recent findings of Kirst *et al.* (2004) showing that in an *E. grandis* back-cross family the genomic regions regulating growth are the same as those controlling lignin content and composition. Indeed, a negative correlation was found between transcript levels estimated for the lignin genes and growth. The most significant correlations were found for *F5H*, *C4H*, *C3H*, *COMT*, *CCoAOMT* and *CAD* (*CCR* was not analysed). It is worth noting that, as in the tobacco over-expressing *Eg*MYB2, S units were more abundant in slow-growing trees (38% increase) compared with fast-growing trees. It is possible that higher carbohydrate consumption for more lignin synthesis may have a negative effect on growth rate.

No significant increase in the Klason lignin content relative to the cell wall residue was detected in transgenic tobacco over-expressing *Eg*MYB2, in contrast to what could be expected from cytological analyses (intensity of phloroglucinol staining), and from the degree of activation of lignin biosynthetic genes. One plausible explanation for this apparent discrepancy relies on the very significant increase (40%) in thickening of the xylem cell walls, which has been observed whatever the method used for microscopic inspection. This thickening, assigned to the S2 layer of the secondary cell walls by electron microscopy, might be the consequence of an increase in the content of at least one of the other major constituents of secondary cell wall: cellulose and hemicelluloses, which would result in underestimating the increase in lignin content relative to cell wall residue.

Interestingly, an increase in phloroglucinol staining suggesting the presence of a higher lignin content or related condensed phenolics was observed not only in the walls of xylem cells, but also in the seed testa. In addition, the fact that the testa develops from the integuments of the ovule, and is therefore a maternal diploid tissue, provides an explanation for the complete absence of germination in the most extreme lines. It is likely that an alteration of cell wall composition occurred in the testa, probably preventing the action of hydrolases needed to complete germination and to allow the radicle to emerge.

At the protein level, the presence of a motif conserved in the C-terminal region of *Eg*MYB2 from *Eucalyptus*, *Poptr*1:4971 from cottonwood and *At*MYB83 from Arabidopsis may indicate functional similarity between these proteins and therefore define a new subgroup of MYB transcription factors. It is worth noting that neither the Arabidopsis MYB gene *At*MYB83 (nor its close sequence *At*MYB46) has been assigned any position among the subgroups defined in the Arabidopsis MYB family (Kranz *et al.*, 1998). This motif was not found in the C-terminal domain of *Pt*MYB4, a MYB gene isolated from a gymnosperm (*Pinus taeda*), highly expressed in xylem and involved in the regulation of lignification (Patzlaff *et al.*, 2003). When over-expressed in transgenic tobacco, both *EgMYB2* and *PtMYB4* act as transcriptional activators of genes committed to the lignin biosynthetic pathway, suggesting that their DNA-binding domains have similar selectivity. The increase in lignin content was higher in *PtMYB4* than in *EgMYB2* over-expressing plants. This might be due, at least in part, to the higher strength of the promoter used (double 35S CaMV) to direct *PtMYB4* expression compared with the single 35S CaMV used to drive *Eg*MYB2 expression. The relative weakness of the latter in xylem has been reported in other studies (Franke *et al.*, 2000). It should also be noted that ectopic expression of *PtMYB4* induces lignification in some cell types that normally do not lignify, such as cells from the pith. Such ectopic lignification was not observed in *EgMyb2*$^{+}$ plants, although in some transformants a higher number of lignified fibres were noticed. Although *Pt*MYB4 and *Eg*MYB2 share a number of common features, they also show some differences, suggesting that they are not functional homologues when working in their natural species. For instance, we have shown in *EgMyb2*$^{+}$ plants that among the most highly activated genes were those encoding enzymes responsible for control of the S/G ratio. Such a role would be unlikely for *Pt*MYB4, as lignins from pine do not contain S units.

Together, these results strongly suggest that *Eg*MYB2 is a positive regulator of secondary cell wall formation and lignin biosynthesis. Moreover, its co-localization with a QTL for lignin content renders it a good candidate for controlling lignin profiles that could be exploited in *Eucalyptus*-breeding programmes.

Experimental procedures

Recombinant DNA methods

Routine DNA methods were used according to Sambrook *et al.* (1989). DNA sequencing was performed with an ABI Prism 3700 DNA sequencer, using the ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit (Amersham Pharmacia Biotech, Orsay, France).

cDNA library screening

Approximately 600 000 pfu of an amplified cDNA library from *E. gunnii* xylem in lambda ZAPII (Lacombe *et al.*, 1997) were blotted onto nitrocellulose membranes following the protocol recommended by the manufacturer (Stratagene, La Jolla, CA, USA), and screened using a consensus 38-mer oligonucleotide [5′-tkccmggaagracmgayaatgaaatcaagaaytaytgc] corresponding to a highly conserved motif in the R3 domain of MYB factors [PGRTDNEIKNYWN] (Jackson *et al.*, 1991), labelled with ^{32}P-ddATP (Amersham) and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Meylan, France). Filters were hybridized overnight at 42°C in 5 × SSPE, 0.25% dry milk powder and 0.05% SDS. Washes were performed at 42°C in 2 × SSC, 0.1% SDS. Plaque-purified positive clones were converted into phagemids (pBluescript SKM13+) following Stratagene's instructions.

RNA isolation

Total RNA was extracted from various *Eucalyptus globulus* tissues harvested on 7-month-old glasshouse-grown plants as recommended by Southerton *et al.* (1998). Total RNA was extracted from leaves of mature glasshouse-grown wild-type and transgenic tobacco plants using the Extract-all kit (Eurobio, Paris, France). In both cases, total RNA was treated with Rnase-free Dnasel (Invitrogen, Cergy Pontoise, France) and purified on columns (Qiagen, RNA Mini Kit). RNA quality and quantity were checked by agarose gel and spectrophotometry.

Quantitative real-time RT-PCR

First-strand cDNA was synthesized from 1 µg RQ1 DNase-treated total RNA in a 20 µl reaction mixture containing 500 ng oligo dT_{12-18}, 0.5 mM dNTPs, 1 µl RNasin and 200 U SuperScript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen). After incubation at 42°C for 1 h and at 65°C for 15 min, the cDNA was purified on Sephadex G50 columns in a 100 µl final volume. 2 µl cDNA was used as template in a quantitative real-time PCR assay (15 µl) performed on the LightCycler Instrument using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I reaction mix (Roche Applied Science, Meylan, France). After a initial denaturation step of 8 min at 95°C, 45 cycles of 15 sec at 95°C, 10 sec at 56–60°C (0–4°C below melting temperature, T_m) and 12 sec at 72°C were performed. Amplification specificity was checked by melting-curve analysis, and PCR efficiency was determined using standard curves constructed with serial dilutions of PCR products as templates. Actin was used as internal control for tobacco leaf samples. The amount of *EgIDH* transcript checked in different tissues of *Eucalyptus* exhibited cycle threshold values (C_t) of 20.13 cycles (mean) ± 0.51 (standard deviation), within the experimental error range of real-time PCR. Therefore *IDH* expression does appear constitutive in the different *Eucalyptus* tissues examined and was used as an internal

control. Quantification of expression ratios was performed according to the mathematical model developed by Pfaffl (2001). Primers and amplicon sizes: *Eucalyptus*, *EgMYB2* (152 bp; upper 5'-gcggatggagattctgtaca, lower 5'-aacgcccttccctactaaga); *EglDH* (115 bp; upper 5'-ctgctggaatctggtatgaaca, lower 5'-tcactctggacatctccatca); *Tobacco* – in the case of multigene families primers have been designed to hybridize to all genes of the class postulated to have a role in lignin biosynthesis: *PAL* (94 bp, primers common to the two class I genes AB008200, X78269, upper 5'-gacaaagtgttcacagcaatg, lower 5'-taacagatwggaagaggagca); *C4H* (124 bp, D. Werck-Reichhart, IBMP, Strasbourg, France, personal communication, upper 5'-tcaacacaatggtggaatgc, lower 5'-actttgggacgtttggttca); *4CL* (89 bp, primers common to the two class I and class II genes U50845, U50846, upper 5'-cttctcaaccatcccaacatt, lower 5'-ctaacaacaaaagccactgga); *HCT* (127 bp, AJ507825, upper 5'-ggctgccaatccatgatgct, lower 5'-gcaacagattgactgccatca); *C3H* (112 bp, primers common to two very close genes; C. Chapple, Purdue University, IN, USA, personal communication, upper 5'-tggctgaggtgatcaagaac, lower 5'-tatgggaggttggggaagtc); *CCoAOMT* (96 bp, primers common to the four class I genes U38612, U62734, U62735, U62736, upper 5'-acaccctatggaatggatca, lower 5'-ccttgttgagttccaatacga); *F5H* (93 bp, primers common to two genes (unpublished data), upper 5'-gaaactctacgacttcaccc, lower 5'-tgactttgccggaatatggt); *COMT* (132 bp, primers common to the two class I genes X74452, X74453, upper 5'-cctgcaaatgggaaggtgat, lower 5'-cagtcctttctttgcctcct); *CAD* (142 bp, primers common to the two very close genes X62343, X62344, upper 5'-ctcgggagaaagagcatcac, lower 5'-cctctccattgcagtgttga); *CCR* (139 bp, C. Halpin, University of Dundee, UK, personal communication, upper 5'-atgtgacgaagccaagggtaa, lower 5'-gtaggaattggaaggtgacct); *Actin* (139 bp, consensus primers to all tobacco constitutive actin genes, upper 5'-attgtkctcagtggtggctc, lower 5'-cctccaatccagacactgta).

*Expression of GST-Eg*MYB2 *in* E. coli

The *EgMYB2* cDNA was recovered from the bluescript plasmid pBSK-*Eg*MYB2 using *Eco*RI and *Xho*I and directionally cloned into the expression vector pGEX-5X-1 (Amersham-Pharmacia). The in-frame fusion 3' to the *Glutathion S-Transferase* (*GST*) gene was checked by sequencing. The resulting plasmid was introduced in *E. coli* strain BL21 and induction of the GST-*Eg*MYB2 fusion protein was realized by adding isopropyl β-D-thiogalactoside (Sigma-Aldrich, St. Quentin, France) to a final concentration of 0.1 mM. After growth at 20°C for 6 h, cells were lysed in buffer: 20 mM Tris pH 7.5, 1 mM EDTA, 10% glycerol, 0,1% NP40, 100 mM PMSF, 10 μg ml^{-1} leupeptin; 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 2 mg ml^{-1} lysozyme, 5 U DNaseI (RQ1, Promega, Charbonnières, France). The fusion protein was purified from the soluble phase using glutathione–sepharose 4B matrix following the supplier's instructions (Amersham-Pharmacia). Protein concentration was estimated with Bradford reagent (Bio-Rad, Marnes la Coquette, France) and proteins extracts were analysed by SDS–PAGE and Western blotting using a mouse primary antibody anti-GST and a secondary antibody (IgG anti-anti-GST of mouse) conjugated with peroxidase.

Electrophoretic mobility shift assay

Vectors containing the *EgCAD2* (Feuillet *et al.*, 1995) or *EgCCR* (Lacombe *et al.*, 2000) promoters were used to amplify by PCR the [−203–129] *EgCAD2* regulatory fragment with the upper 5'-tctcgagatggctaaaaagcaagtcttgc-3' and lower primer 5'-ggcgaaaagtgacactcgagcaagc, and the [−119–70] *EgCCR* regulatory fragment with the upper primer 5'-ggtctcgagaggggagcg and the lower primer 5'-gactcgagttaccaaga, all the primers containing *Xho*I restriction sites. The PCR products were cloned in pGEM-T vector (Promega) and checked by sequencing. After *Xho*I digestion the resulting *EgCAD2* and *EgCCR* regulatory fragments were purified on agarose gels and 100 ng were 3'-end labelled for 30 min at 37°C with the Klenow fragment of DNA polymerase I (5 U) in a final volume of 20 μl with 33 μM of each of dATP, dTTP, dGTP and 4 μl α-^{32}P[dCTP] (10 mCi ml^{-1}). The labelled fragments were purified on a 4.8% polyacrylamide gel and eluted in water overnight at 4°C. Binding reactions were performed in a total volume of 25 μl, with 5000–10 000 cpm labelled DNA fragments (20–30 fmol), 30–100 ng purified GST-*Eg*MYB2 or GST alone, 10 mM Tris–HCl pH 8, 150 mM NaCl, 10% glycerol and 500 ng poly dIdC-poly dIdC (Gibco-BRL, Paris, France). For competition experiments, non-radioactive competitors were added to a 100-fold molar excess ratio relative to the probe. The binding reactions were incubated for 30 min at room temperature and analysed on a 4.8% polyacrylamide gel as previously described by Lacombe *et al.* (2000).

Binary constructs for transformation

The *EgMYB2* cDNA was subcloned as a *Kpn*I–*Xba*I fragment into the pGEM-T vector (Promega) and the sequence was checked. The 1.4 kb cDNA fragment was cloned into the binary vector pJR1 (Piquemal *et al.*, 1998) under the control of the 35S CaMV promoter, generating the *EgMyb2*$^{+}$ construct. For downregulation we generated a dominant negative construct. The plasmid pGEMT-*EgMYB2* (mentioned above) was digested with *Kpn*I and *Pst*I and the resulting 0.5 kb fragment corresponding to the DNA-binding domain (DBD) was placed under the control, the 35S *CaMV* promoter in an intermediary vector pBOB13 (EL, unpublished data). After digestion by *Hind*III and *Eco*RI, the [*35S promoter-EgMYB2 DBD-Nos terminator*] cassette was inserted into the binary vector pBin19, leading to the construct *Eg*MYB2^{-}. Using the freeze–thaw procedure (Holsters *et al.*, 1978), the constructs *EgMyb2*$^{+}$ and *EgMyb2*$^{-}$ were introduced into *Agrobacterium tumefaciens* strain LBA4404 for stable transformation, and/or into strain C581pCH3 for transient co-transfection experiments.

Co-transfection experiments

Co-transfection experiments were performed essentially according to the method of Yang *et al.* (2000). *Agrobacterium* strains C581pCH3, containing either a binary effector plasmid or a reporter construct, were co-infiltrated in near fully expanded leaves of tobacco plants using a 1 ml syringe. After agro-infiltration, plants were maintained in a growth chamber at 22°C under 16 h light for 3 days. Quantitative GUS assays were carried out on the infiltrated zone using 4-methylumbelliferyl-β-D-glucuronide as substrate (Jefferson, 1987). Protein concentrations were determined by the Bradford method (Bio-Rad). GUS activities were estimated as the mean of three independent assays, each containing at least three replicates.

Tobacco plant transformation

Tobacco (*Nicotiana tabacum* cv. Samsun NN) was transformed by a modification of the leaf-disc method (Horsch *et al.*, 1985). Regeneration and propagation procedures were as described by Piquemal *et al.* (1998). More than 15 independent tobacco transformants were generated for each construct, propagated *in vitro* and transferred to the glasshouse. The presence of the transgene was confirmed by PCR on genomic DNA using specific primers for kanamycin

resistance and for MYB genes (described above). F_1 seeds obtained by self-pollination of transformants were harvested and selected further on germination medium containing kanamycin (500 μg ml^{-1}). The sterilization treatment was for 2 min in ethanol 70% followed by 5 min NaOCl, 5%.

Microscopy and cell imaging

Transverse sections (100 μm thick) in the lower part of tobacco stems were obtained using a vibratome (Microcut H1250; Energy Beam Science Inc., St. Louis, MO, USA). They were observed either under UV excitation (excitation filter BP 340-380 nm, suppression filter LP 430 nm) or under bright-field after phloroglucinol (Wiesner reagent) and Maüle staining for lignin visualization. Other samples, dehydrated in ethanol, were embedded in Spurr's epoxy resin. Semi-thin (1–2 μm) and ultrathin sections (90 nm) were obtained using an ultramicrotome (Reichert UltraCutE; Leica Microsystems, Rueil Malmaison, France). Semi-thin sections, mounted on glass slides, were observed in confocal microscopy (LSM SP2; Leica) using the 488 nm ray line of the argon laser. The emitted light was collected between 500 and 550 nm. Other semi-thin and ultrathin sections were treated by $KMnO_4$ and observed either under bright-field or with an electron microscope at 80 kV (Hitachi, Naka, Japan). In optical microscopy, images were acquired using a CCD camera (Color CoolView; Photonic Science, Milham, UK). The thickness of the xylem cell walls was determined by image analysis (IMAGE PROPLUS software; Media Cybernetics, Silver Spring, MD, USA). Micrographs of seeds were acquired with a stereomicroscope (MZFLIII; Leica) equipped with a COD camera (DC200; Leica).

Lignin analysis

Basal parts of stems of control and transgenic mature plants were harvested and frozen in liquid nitrogen. After lyophilization the stem samples were ball-milled to a fine powder and extracted as previously described by Piquemal *et al.* (1998) for subsequent determinations of Klason lignin contents using the Klason technique (Dence, 1992). Thioacidolysis was performed using the method of Lapierre *et al.* (1986).

Gene mapping and QTL analysis

Genetic mapping of *EgMYB2* was performed using an interspecific F_1 hybrid progeny (201 full sibs) between *E. urophylla* (female) and *E. grandis* (male) (Verhaegen and Plomion, 1996). Conformation polymorphism was detected using the SSCP technique as described previously by Gion *et al.* (2000) with specific primer pairs (upper 5′-tccaatccacaagacatagc, lower 5′-gtgggggaacagaaaactcg). In comparison with the *E. gunnii EgMYB2* sequence, four and six nucleotides were different in the *E. urophylla* and *E. grandis* sequences, respectively, indicating that both parents were heterozygous at this locus. Using a migration temperature of 15°C for 15 h electrophoresis, four bands were obtained, segregation of which in the progeny was consistent with the patterns observed in the parents. In order to map the newly genotyped SSCP *EgMYB2* marker to linkage maps previously established using RAPD markers for both parents (Verhaegen and Plomion, 1996; Verhaegen *et al.*, 1997), linkage analysis was performed using the MAPMAKER programme (Whitehead Institute, Cambridge, MA, USA) with a minimal linkage LOD of 6 and a maximum recombination fraction θ of 0.30. Assessments of lignin content using the Klason method was made on the 201 progenies used for establishing both genetic maps at 62 months, which corresponds to harvest age in commercial *Eucalyptus* plantations. The lignin content presented a normal distribution in the progeny studied. The QTL analysis was performed on each parental map under the back-cross model. Both the interval-mapping methods implemented in MAPMAKER/QTL (maximum likelihood) and OGENE (linear least squares; Whitehead Institute) with a threshold of 1.7 were used to declare a putative QTL for lignin content.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ576023.

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